

APPLE PHYTOCHEMICALS IN THE PREVENTION OF BREAST CANCER:  
EFFECTS ON CELL PROLIFERATION AND APOPTOSIS

A Dissertation  
Presented to the Faculty of the Graduate School  
of Cornell University  
in Partial Fulfillment of the Requirements for the Degree of  
Doctor of Philosophy

By  
Dana Lynn Felice  
May 2009

© 2009 Dana Lynn Felice

APPLE PHYTOCHEMICALS IN THE PREVENTION OF BREAST CANCER:  
EFFECTS ON CELL PROLIFERATION AND APOPTOSIS

Dana Lynn Felice, Ph.D.

Cornell University 2009

Among women in the United States, breast cancer is the most frequently occurring cancer and is the second-leading cause of cancer deaths. Approximately 30% of cancer risk is estimated to be attributed to diet. Apple consumption has been correlated with reduced breast cancer risk as well as more favorable prognostic factors. The first objective was to develop a more efficient methylene blue assay for cell counting which could be applied to a broader range of cell densities and various cell culture plates. A linear relationship ( $r^2 > 0.99$ ) was established between cell number and absorbance at 570 nm wavelength when the modified methylene blue assay was applied to three cell lines plated in a broad range of cell densities in 4 different types of culture plates. This modified methylene blue assay is a powerful tool for any application requiring a versatile, efficient, and accurate method of cell counting, especially in the research of natural products, bioactive compounds, phytochemicals, functional foods and nutraceuticals. The second objective was to determine whether whole apple extracts and purified compounds from apples interfered with IGF-I-mediated signal transduction pathways in MCF-7 human breast cancer cells. Quercetin-3-Glucoside (Q-3-G) proved to be surprisingly effective, inhibiting IGF-I-induced MCF-7 cell proliferation more potently than FBS-stimulated proliferation, as well as more potently than quercetin inhibited cell growth. Q-3-G blocked cell cycle progression and induced apoptosis via down-regulated Akt phosphorylation, Cyclin D1 and CDK-4 expression, and increased Caspase-9 and PARP cleavage. The results

help to explain the mechanisms by which apple consumption may reduce breast cancer risk. Additionally, such studies of purified compounds contribute to the understanding of cancer cell behavior and may inform future endeavors in chemotherapeutic drug design. The third objective was to determine the effects of apple phytochemical extracts on cell proliferation and apoptosis in mammary tissues in DMBA-treated Sprague-Dawley rats *in vivo*. The expression of PCNA, BrdU-labeled cells, Cyclin D1 and Bcl-2 decreased, and Bax expression and apoptosis increased in the apple treated groups. These results demonstrate the potent capacity of fresh apples to suppress promotion and progression of DMBA-initiated mammary carcinogenesis in rats.

## BIOGRAPHICAL SKETCH

Dana Felice grew up in New Jersey with her parents and three sisters. She earned a Bachelor of Science degree from Marist College in Poughkeepsie, NY. It was there that she majored in Environmental Science under the tutelage of Dr. Zofia Gagnon. Dana received the Central Hudson Energy Group Inc. Award for Excellence in Environmental Science upon graduation. While at Cornell, she majored in Environmental Toxicology and carried out her research on apple phytochemicals and breast cancer prevention under the guidance of Dr. Rui Hai Liu. Dr. Andrew Yen and Dr. Dan Brown served as committee members for her minors in cancer biology and nutrition, respectively. Dana and her husband met at Cornell, where they were married in August of 2007. They currently reside in Chicago, IL.

*Dedicated to Mom and Dad.*

*To thank you for your love and support seems an understatement. What a gift to grow up knowing you are loved unconditionally. Thank you for being such beautiful examples of what it means to be kind, loving, and generous; for the sacrifices you made for the sake of my education; for encouraging me to “reach for the stars” (“per aspera, ad astra!”); for being the best parents anyone could ask for.*

*I love you up to the sky!*

## ACKNOWLEDGMENTS

I would like to express my gratitude to many wonderful people who have influenced and shaped my education. Thank you to my committee chair, Dr. Rui Hai Liu, for supporting my graduate education and research at Cornell. Thank you also to Dr. Andy Yen and Dr. Dan Brown, my minor advisors. I appreciate the insights and encouragement you all offered over these years. Working with you was such a privilege. I absolutely must thank Dr. Zofia Gagnon, my academic advisor and mentor during my years at Marist College. From day one, you were instrumental in my successful completion of undergraduate coursework and research as well as my acceptance into graduate school. Thank you to Drs. Laura Arwood, Richard Feldman, Raymond Kepner, Richard LaPietra, Thomas Lynch, Theresa Snyder and Michael Tannenbaum. You helped me to get the most out of my Marist education! Thank you to Mrs. Nancy Estevez and Mr. Frank Setlock from Pope John XXIII High School in Sparta, NJ. You both inspired and encouraged my love for science. To all of these influential teachers from high school through graduate school, I have such great respect and appreciation for you.

Thank you to Cindy Wright-Uhrovic and Sharon Johnston, who both went above and beyond their job descriptions on a daily basis in order to make the entire process of graduate school run so smoothly. There simply isn't a big enough word to sufficiently express my gratitude! Many thanks to the Food Science Department faculty and staff, for their help and support over the years. To my labmates whom I consider colleagues, teachers, and friends, thank you: Kelly Wolfe, Lena Vayndorf, Jun Yang, Yi-Fang Chu, Kafui Adom, Chang-Shu Liu, Hyungeun Yoon, Neal O'Karter, Jeanelle Boyer, Natt Thudnatkorn, Xing-Mei Kang, and especially Jia Ren Liu and Jie Sun for

imparting so much of their knowledge. Many thanks to those who collaborated on various aspects of this research: Jia Ren Liu, Jie Sun, Sharon Johnston and Xing-Mei Kang. Thank you to James Smith from the Biomedical Sciences Flow Cytometry Core Laboratory at Cornell for running the flow cytometry samples included in this manuscript and to Kelly Wolfe for running the CAA assay. I am grateful for the opportunity to have studied at Cornell, where the people and the experiences helped me to grow both professionally and personally.

To my Marist girls, Heather Bachman, Maureen Connolly, Kathryn Docherty and Lynne Leach, thank you for your friendship over the years. A special thanks to Kathryn and Lynne for their moral support as we each experienced graduate school at three different universities. To my fellow Toxies, Tone Helland, Rachelle Kosoff and Bojeong Kim, and my neighbors in the Big Yellow House, Evan Schwartz and John DeFranco, thank you for your friendship. To Carolyn Kalsow, thank you for your listening ear and your thoughtful advice.

Thank you to my beautiful family for loving me, believing in me, and encouraging me to work hard to achieve my dreams. Thanks Mom and Dad (Ginni and Philip), Tracy and Joe, Stephanie, Luca and Lily, and Christa. You are the most loving, generous and admirable people I know, who inspire me every day! I love you! Thank you to my husband, Joe Podwol. I am so grateful to have experienced graduate school together. Thank you for believing in me and my research, even though it meant living 700 miles apart for the first eleven months of our marriage. You are amazing! I love you!

Finally, thank you God for the many blessings in my life!



## TABLE OF CONTENTS

Biographical Sketch	iii
Dedication	iv
Acknowledgments	v
List of Figures	viii
List of Tables	x
Chapter 1. Literature Review. Diet and Breast Cancer: The Roles of Apple Phytochemicals and IGF-I Receptor Signaling	1
Chapter 2. A Modified Methylene Blue Assay for Accurate Cell Counting	88
Chapter 3. Quercetin-3- $\beta$ -D-Glucoside Inhibits IGF-I-Mediated MCF-7 Cell Survival by Blocking Cell Cycle Progression and Inducing Apoptosis via Down-Regulation of Akt Activation	117
Chapter 4. Fresh Apples Inhibit Proliferative Activity and Induce Apoptosis in DMBA-Initiated Mammary Tissues in Rats	173

## LIST OF FIGURES

<b>Figure 1.1.</b> IGF-I Receptor-Mediated Signaling Pathways.	46
<b>Figure 2.1.</b> Methylene Blue Staining Kinetics.	99
<b>Figure 2.2.</b> Methylene Blue Elution Kinetics.	100
<b>Figure 2.3.</b> Relationship of cell number to relative absorbance in three cell lines and four different culture plates.	101
<b>Figure 2.4.</b> Number of Caco-2 cells determined by the trypan blue cell counting assay compared to the number of cells initially plated.	104
<b>Figure 2.5.</b> Comparison of cell growth curves determined by the trypan blue assay and the modified methylene blue assay in both human Caco-2 colon cancer cells and HepG2 liver cancer cells <i>in vitro</i> .	106
<b>Figure 2.6.</b> Applications in phytochemical research: Antiproliferative activity.	108
<b>Figure 3.1.</b> Effects of whole apple extracts and pure phytochemicals on MCF-7 cell proliferation induced by FBS or by IGF-I.	128
<b>Figure 3.2.</b> Cytotoxicity of Quercetin and Quercetin-3- $\beta$ -D-Glucoside (Q-3-G).	129
<b>Figure 3.3.</b> Cell Proliferation: Quercetin and Q-3-G.	130
<b>Figure 3.4.</b> Cellular Antioxidant Activity (CAA) Assay.	133
<b>Figure 3.5.</b> Effect of Q-3-G on cell cycle distribution of IGF-I-stimulated MCF-7 cells.	135
<b>Figure 3.6.</b> Effects of Q-3-G on IGF-I Receptor expression and phosphorylation.	137
<b>Figure 3.7.</b> Effects of Q-3-G on Akt expression and phosphorylation.	139
<b>Figure 3.8.</b> Effect of Q-3-G on Cyclin D1 expression.	140
<b>Figure 3.9.</b> Effect of Q-3-G on CDK-4 expression.	141
<b>Figure 3.10.</b> Effect of Q-3-G on expression of p21, p27 and PCNA.	142
<b>Figure 3.11.</b> Effect of Q-3-G on Sub-G0 peak.	144

<b>Figure 3.12.</b> Acridine Orange/Ethidium Bromide (AO/EB) staining of MCF-7 cells treated with Q-3-G.	145
<b>Figure 3.13.</b> Effect of Q-3-G on Caspase-9 cleavage.	146
<b>Figure 3.14.</b> Effect of Q-3-G on PARP cleavage.	147
<b>Figure 3.15.</b> Effect of Q-3-G on Bcl-2 expression.	148
<b>Figure 4.1.</b> Effects of varying doses of apple extracts on body weight of rats.	180
<b>Figure 4.2.</b> Effects of varying doses of fresh apple extracts on BrdU labeling in rat mammary gland cells <i>in vivo</i> .	181
<b>Figure 4.3.</b> Effects of varying doses of fresh apple extracts on PCNA expression in rat mammary gland tissues <i>in vivo</i> .	183
<b>Figure 4.4.</b> Effects of varying doses of fresh apple extracts on expression of Cyclin D1 in rat mammary gland tissues <i>in vivo</i> .	184
<b>Figure 4.5.</b> TUNEL Assay: Effects of varying doses of fresh apple extracts on induction of apoptosis in rat mammary gland tissues <i>in vivo</i> .	185
<b>Figure 4.6.</b> Effects of varying doses of fresh apple extracts on Bcl-2 expression in rat mammary gland tissues <i>in vivo</i> .	187
<b>Figure 4.7.</b> Effects of varying doses of fresh apple extracts on Bax expression in rat mammary gland tissues <i>in vivo</i> .	188

## LIST OF TABLES

<b>Table 2.1.</b> Recommended cell numbers and reagent volumes per well for methylene blue assay.	95
<b>Table 3.1.</b> Anti-Proliferative and Anti-Oxidant Activities of Quercetin and Quercetin-3- $\beta$ -D-Glucoside (G-3-G) in MCF-7 cells.	131

## CHAPTER 1

### DIET AND BREAST CANCER: THE ROLES OF APPLE PHYTOCHEMICALS AND IGF-I RECEPTOR SIGNALING

#### I. BREAST CANCER

In the United States, breast cancer is the most frequently occurring cancer among women, accounting for an estimated 26% of new diagnoses in 2008, and is the second-leading cause of cancer deaths among women, accounting for 15% of cancer deaths estimated in 2008 (estimates exclude basal and squamous cell skin cancers and *in situ* carcinoma except bladder) (American Cancer Society, 2008). The American Cancer Society predicts ~185,000 new cases and more than 40,000 deaths due to breast cancer in 2008. While advances in treatment are being made, cancer is still considered a preventable disease since many significant causes are exogenous, such as viruses and carcinogenic chemicals. As discussed below, epidemiology studies have linked a diet high in fruits and vegetables, including apples, with reduced breast cancer risk, and cell and molecular studies are beginning to reveal the many and varied anti-cancer properties of the dietary phytochemicals. Therefore, research in the area of breast cancer prevention is necessary and valuable.

#### II. DIET AND CANCER

**A. Cancer is a Preventable Disease.** Cancer has long been viewed as a preventable disease. In 1981, Doll and Peto calculated that ~35% of cancer deaths in the U.S. were due to dietary factors (Doll and Peto, 1981). In 1995, Willett concluded that although the body of knowledge on the subject had grown immensely since 1981, the estimate still had not changed drastically (Willett, 1995), offering a range of 20 to

42% of cancer deaths being attributed to diet. Peto (2001) revisited the subject, discussing the major, and for the most part avoidable, causes of cancer. The observation that immigrants adopt the cancer risk of the native population of their new homeland offered insights into the environmental causes of cancer (Dunn, 1977; McMichael *et al.* 1980; Stanford *et al.* 1995). The association between tobacco use and cancer was another critical discovery (Doll and Hill, 1950; Wynder and Graham, 1950). Up to 60% of cancer deaths among smokers can be attributed to their tobacco use (Peto *et al.* 1994). Reproductive and hormonal factors play a significant role in certain cancers, such as the contribution of lifetime estrogen exposure (e.g. ages of menarche, first childbirth, and menopause; hormone replacement therapy, etc.) to breast cancer risk (Kelsey, Gammon and John, 1993). Many cancers are associated with infectious pathogens such as *Helicobacter pylori* and stomach cancer (IARC, 1994a), human papillomaviruses (HPVs) and cervical cancer (Walboomers *et al.*, 1999), hepatitis B virus and hepatocellular carcinoma (HCC), and hepatitis C virus and HCC (IARC, 1994b) while occupational exposures have been linked with certain cancers such as asbestos and mesothelioma (Peto *et al.*, 1999). Being overweight has been shown to increase overall cancer risk (Josefson, 2001).

**B. Diet Accounts for up to 30% of Cancer Risk.** Peto also discussed 3 important studies that attempted to quantify the contribution of diet to cancer risk (COMA, 1998; Glade, 1997; Doll and Peto, 2003). While the 3 studies reported a contribution of about one third, “opinions still differ on the strength of the evidence” (Peto, 2001). Taken together, Peto (2001) calculates that after eliminating the avoidable causes of smoking, known infections, alcohol, sunlight, air pollution, lack of exercise and overweight, diet may account for 10 to 30% of cancer deaths.

**C. Fruits, Vegetables, and Cancer Risk.** Fruit and vegetable consumption has been consistently linked to reduced risk of cancer, as demonstrated by an analysis

of approximately 200 epidemiology studies relating diet and incidence of various cancers (Block *et al.*, 1992). Steinmetz and Potter (1996) reviewed 20 cohort and 174 case-control studies which evaluated associations between fruit and vegetable consumption and various cancers. Of the 20 cohort studies, 19 found inverse associations between some category of fruit and/or vegetable and some type of cancers; 12 of these associations were statistically significant. The most consistent protective effect of fruit and vegetable consumption was found to be against lung cancer. Of the 174 case-control studies evaluated in the paper, only prostate cancer did not show a significant inverse relationship with any category of fruit or vegetable consumption, although 5 out of 5 studies showed a non-significant inverse association. A significant inverse association was found, however, in more than 80% of the studies for cancers of the stomach, esophagus, lung, oral cavity and pharynx, rectum, bladder, cervix, endometrium, and larynx (Steinmetz and Potter, 1996).

Studies of stomach cancer, lung cancer, and colon cancer were quite convincing. Of the 31 studies which looked at stomach cancer, 28 (93%) found a significant inverse relationship, 2 (7%) found a non-significant inverse relationship, and one study did not report the significance of their results. The break-down of fruit and vegetable associations with stomach cancer was as follows: vegetables: 11 out of 11 studies found an inverse association; 17 fruit studies found 14 inverse and 3 null; raw vegetables: 10 out of 10 inverse; legumes: 7 inverse and 2 positive; allium vegetables: 9 inverse, 1 null, 1 positive; green vegetables: 8 out of 8 inverse; carrots: 7 inverse, 1 null, 1 positive; tomatoes: 9 inverse, 1 null, 1 positive; citrus fruits: 11 inverse and 1 null association. Of the 13 studies which evaluated associations between fruit and vegetable intakes and lung cancer, 11 (85%) found significant inverse relationships and 1 (15%) found statistically non-significant inverse relationships. 7 out of 7 studies found an inverse relationship between vegetable intake and lung

cancer; 8 out of 8 found an inverse association with fruit, 9 out of 9 found an inverse association with green vegetables, and 4 out of 4 studies found an inverse association with tomatoes. Out of 7 studies that measured intake of carrots, 6 associations were inverse and 1 was found to be null (Steinmetz and Potter, 1996).

Out of 21 studies which evaluated fruit and vegetable intake and colon cancer, 15 (79%) found significant inverse relationships, 4 (21%) found non-significant inverse relationships, and 1 study did not report the significance of their results. The breakdown by food group was as follows: vegetables: 9 inverse associations and 1 positive association; fruit: 5 inverse, 2 null and 1 positive; raw vegetables: 3 inverse, 1 positive; cruciferous vegetables: 8 inverse, 3 null and 1 positive; legumes: 1 inverse, 2 null, 2 positive; allium vegetables: 4 inverse, 1 null, 1 positive; green vegetables: 4 inverse, 1 null; carrots: 4 inverse, 1 null, 2 positive; tomatoes: 4 inverse, 2 positive; citrus fruits: 2 inverse, 1 null and 3 positive (Steinmetz and Potter, 1996).

Out of the 174 case-control and 20 cohort studies evaluated in this paper, vegetables, raw vegetables, and fruit are the groups that demonstrated the most convincing protective effects against cancer overall: 81% (55 out of 68 studies) showed an inverse association between vegetables and cancer, 85% (33 out of 39 studies) showed an inverse association between raw vegetables and cancer, and 63% (29 out of 46 studies) showed an inverse association between fruit and cancer. In many of the studies, groups with the lowest intake of fruits and vegetables had as much as twice the cancer rate of populations with the highest intake (Steinmetz and Potter, 1996).

While the anti-cancer properties of fruits and vegetables are attributed to the phytochemicals, the non-nutrient bioactive components of the plant material, studies attempting to show a link between particular phytochemicals and cancer risk have not



been as convincing. For example, in their review of dietary flavonoids, Ross and Kasum (2002) found sufficient data to support the hypothesis that high fruit and vegetable intake can decrease cancer risk. However, they note that epidemiological studies attempting to make the same correlation with flavonoids specifically are not conclusive (Ross and Kasum, 2002).

The Zutphen Elderly Study, for instance, found no association between cancer mortality and flavonol or flavones intake based on food frequency questionnaires (Hertog *et al.* 1993a; Hertog *et al.* 1993b; Arts *et al.* 2001). Arts *et al.* (2001) looked at the same cohort ten years later, and concluded that catechins from non-tea sources slightly reduced lung cancer incidence, yet tea catechins did not (Ross and Kasum, 2002). A study in the Netherlands followed rather large cohorts (58,279 men and 62,573 women) for four years and found no association between flavonol or flavones intake and incidence of several forms of cancer (stomach, colon, lung) (Goldbohm *et al.*, 1995). In a Finnish cohort study of 9,959 men and women, flavonoid intake correlated with decreased incidence of total cancers (Knekt *et al.*, 1997). A case-control study in Uruguay analyzed dietary data from a group of individuals and hospitalized controls, reporting reduced cancer incidence associated with carotenoid, glutathione, flavonoid, vitamin E, and total fruit and vegetable intake (DeStephani *et al.*, 1999). In a case-cohort analysis attempting to relate antioxidant and folate intake with male lung cancer risk, it was determined that lutein+zeaxanthin,  $\beta$ -cryptoxanthin, folate and vitamin C exerted protective effects against lung cancer incidence, while  $\alpha$ -carotene,  $\beta$ -carotene, lycopene and vitamin E did not (Voorrips *et al.*, 2000).

The discrepancies among these epidemiology studies, which show a correlation between high fruit and vegetable intake and reduced cancer risk, but inconclusive results regarding a particular class of phytochemical with cancer risk, may offer important insights. Experimental designs and methods of data collection and

analysis differ, making comparisons across studies difficult. But perhaps more importantly, this phenomenon may indicate the mechanisms by which the fruits and vegetables exert their anti-cancer activities. In other words, it may not be one particular class of phytochemicals that is working alone and is responsible for the protective effects seen. It is becoming clear that the greatest benefits of the phytochemicals are conferred through the additive and synergistic effects of these compounds in our diets (Liu, 2003; Liu, 2004; Liu and Felice, 2007).

### **III. DIET AND BREAST CANCER**

The relationship between diet and breast cancer continues to be extensively studied. Many dietary and lifestyle factors have been associated with breast cancer risk, some more convincingly than others (Holmes and Willett, 2004). All told, the most convincing factors increasing breast cancer risk are alcohol consumption, overweight or obesity, and physical activity. The common link among the three is their influence on circulating estrogens, androgens, and insulin, elevated levels of which are all correlated with increased breast cancer risk. Alcohol consumption increases endogenous estrogen and androgen concentrations (Holmes and Willett, 2004; Hanf and Gonder, 2005; Berrino *et al.*, 2006). As summarized by Holmes and Willett, combined results of 6 large cohort studies demonstrated a 9% increase in breast cancer risk for every 10 g alcohol/day (95% CI = 4 to 13%; beer, wine or liquor). Intervention studies showed increased estrogen levels in both pre- and post-menopausal women with addition of 1 to 2 alcoholic drinks daily. Hanf and Gonder discuss alcohol's abilities to interfere with hormone metabolism (30 g of ethanol daily increased bioavailable estrogens compared to the control group), increase transport of less soluble carcinogens, and damage cell membrane integrity and cell-to-cell

communication (Hanf and Gonder, 2005). Finally, the synthesis of both androgens and estrogens is increased by alcohol consumption (Berrino *et al.*, 2006).

Obesity, a sedentary lifestyle, and a high fat diet lead to metabolic syndrome (having at least 3 of the following 5 symptoms: increased plasma glucose, increased triglycerides, decreased HDL, increased waist circumference and hypertension) (Berrino *et al.*, 2006). Metabolic syndrome may lead to increased androgenic activity and insulin resistance. Increased circulating insulin will increase synthesis of androgens and growth hormone receptor expression, while blocking synthesis of IGFBP1 (Insulin-like Growth Factor Binding Protein 1), IGFBP2 and SHBG (Sex Hormone Binding Globulin), thus rendering IGFs and estrogen more bioavailable. Physical activity appears to confer protective effects by increasing insulin sensitivity, decreasing testosterone, and decreasing IGF-I concentrations. The effect of exercise is so strong that 30 minutes daily of brisk walking cuts risk of breast cancer recurrence by half (Berrino *et al.*, 2006).

**A. Dietary Factors and Breast Cancer.** Many epidemiology studies have investigated the influence of a low-fat, high-fiber diet on estrogen levels and breast cancer risk (Forman, 2007). A high-fat diet encourages re-absorption of estrogens in the intestine by increasing activity of a deconjugating enzyme. A high-fiber diet counters that process and thus more estrogens are excreted rather than reabsorbed into the blood stream. Accordingly, vegetarians were shown to have higher fecal estrogen levels while omnivores had higher urinary estrogen levels (Dos Santos Silva *et al.*, 2002). Two meta-analyses, one in 1999 and one in 2007, concluded that low-fat, high-fiber diets reduced estrogen levels (Wu *et al.*, 1999; Forman, 2007).

Wu *et al.* (1999) analyzed 14 papers that were published between January 1966 and June 1998 that fit their criteria: they included only intervention studies that

reported the level of fat intake, duration of the study, and endogenous estrogen levels before and after intervention or the percent change. Only studies on healthy women were included and not those that involved patients with breast cancer. Ultimately the meta-analysis included 10 studies of premenopausal women and 4 studies of postmenopausal women. In the studies of premenopausal women, the subjects' baseline diets contained 29 – 46% of calories from fat, and changed to a low fat diet (12 – 25% of calories from fat) for 2 to 3 months. The fiber intake changed as well (~30 g/d increase in 4 studies and 2-9 g/day increase in 6 studies). The low fat diet significantly reduced circulating estradiol levels in 2 studies; in 7 studies the decrease was  $\geq 5\%$ , in one study decreased  $< 2\%$ , and in 2 studies estradiol increased but slightly and non-significantly. In the 4 intervention studies of postmenopausal women, fat intake ranged from 10 to 24% of calories for 3 weeks to 5 months. There were large differences in fiber intake among these studies, two of which did not report fiber intake, one of which increased fiber by 2 g/day, and the last whose subjects consumed 35-45 g/d (per 1000 kcal). The results showed a statistically significant decrease in estradiol in 2 studies, a non-significant decrease in one, and a non-significant increase in the last (Wu, Pike and Stram, 1999). The authors' pooled analysis of the data generated the following results: in premenopausal women, estradiol levels changed by -7.4% (95% CI = -11.7% to -2.9%); in postmenopausal women, -23.0% (95% CI = -27.7% to -18.1%); combined, -13.4% (95% CI = -16.6% to -10.0%). Two studies which decreased fat intake the most, to 10 and 12% of calories, had the most dramatic effects. Removing these studies from the pooled analysis changed the results to -6.7% (95% CI = -11.1 to -2.1%) in premenopausal women; -6.2% (95% CI = -13.1 to -1.3%) in postmenopausal women; and -6.6% (95% CI = -10.3 to -2.7%) combined. The authors considered the heterogeneity of the studies, and indeed removing the 2 lowest-fat studies decreased the heterogeneity across studies. Repeating the pooled

analysis accounting for heterogeneity changed the results somewhat: in pre- and postmenopausal women combined, the low-fat diet changed estradiol by -6.8% (95% CI = -13.2 to 0.1%). Ultimately this analysis marginally increased the change in estradiol level but widened the confidence interval such that the result was no longer statistically significant at the  $\alpha = 0.05$  level. Yet from a pragmatic standpoint the outcome still demonstrates a correlation between fat intake and estrogen levels. However the data were analyzed, the results supported the conclusion that reduced fat intake leads to reduced circulating levels of estradiol (Wu *et al.*, 1999).

Although there were differences across studies in the type, timing, and duration of the dietary intervention, and weight loss and physical activity were not considered in many studies, the effect of reduced estrogen levels as a result of a high fiber/low fat diet was seen in girls and in pre- and post-menopausal women (Forman, 2007). The differences were not always significant, but the trend existed. Forman discusses the importance of considering the following in designing future intervention trials investigating risk factors for breast cancer: Among ethnic groups, there are differences in energy expenditure, endogenous hormone levels, average age of menarche, and gene polymorphisms. Estrogen levels are affected by ethnicity, gene polymorphisms, life cycle (e.g. age of menarche and menopause, parity, etc.), normal menstrual cycle variations, physical activity, weight and diet (Forman, 2007, and sources therein).

**B. Fruits, vegetables and breast cancer.** Out of 13 case-control studies that measured associations between fruit and vegetable intakes and breast cancer, 9 (69%) found significant inverse relationships and 4 (31%) found non-significant inverse relationships (Steinmetz and Potter, 1996). Four of those studies measured fruit intake and breast cancer: 3 found inverse associations and one found a positive association. Six studies measured intake of green vegetables: 5 inverse and one null; 4 measured

carrots and found 3 inverse and 1 null; 3 measured citrus fruits: one inverse and 2 positive (Steinmetz and Potter, 1996).

Holmes and Willett (2004) reported that case-control studies associated increased fruit and vegetable consumption with a decreased breast cancer risk, while 8 prospective studies demonstrated weak and non-significant relationships. Rock (2003) reported that the protective effect of fruit and vegetable consumption, in 8 follow-up epidemiology studies, ranged from a 20 to 90% reduction in risk of death from breast cancer. The WHEL (Women's Healthy Eating and Living) Study showed that a diet with increased fruits, vegetables, grains and fiber and decreased fat, without weight loss, had a protective effect against breast cancer (Rock, 2003).

Duncan (2004) concisely summarized the findings of several studies linking fruit and vegetable intake and breast cancer: A review of 19 case-control and 3 cohort studies by the American Institute for Cancer Research showed a protective effect of fruit and vegetable consumption (Glade, 1997). A meta-analysis of 14 case-control and 3 cohort studies found a protective effect of vegetables, but not fruit (Gandini *et al.*, 2000). The Nurse's Health Study found a non-significant reduced risk with higher fruit and vegetable consumption among premenopausal women (5 or more servings: 33% reduced risk vs. 2 or fewer servings) (Zhang *et al.*, 1999). A pooled analysis of 8 prospective studies revealed a non-significant 7% reduction in BC risk among those with the highest fruit and vegetable consumption (Smith-Warner *et al.*, 2001). Duncan concludes the evidence, although not always statistically significant, supports a protective effect of fruits and vegetables against breast cancer risk, citing the proper consideration of various micronutrients and phytochemicals as possible reasons for the inconsistencies among studies (Duncan, 2004).

In contrast to their 1997 report mentioned above, the World Cancer Research Fund and the American Institute for Cancer Research (AICR) were unable to reach conclusions regarding the influence of fruit or vegetable intake on either pre- or postmenopausal breast cancer risk in their 2007 report. The reasons cited were that the data reviewed were inconsistent or of inadequate quality, or too few studies existed (World Cancer Research Fund, 2007).

The non-significant findings of the Pooling Project mentioned above (Smith-Warner *et al.*, 2001) may have much to do with study design (Slattery, 2001). Among the 8 cohort studies analyzed, there were variations in the questionnaires in number of different food categories listed as well as which specific food items were included. For example, many studies in the Pooling Project did not include such commonly consumed fruits as apples, bananas and oranges. Not only does this create differences between studies, but the lists could have been incomplete compared to what the subjects were actually consuming. In the case where a subject left a question unanswered, the response was recorded as non-consumption of that particular food; this can affect energy intake analysis as well as the final associations made. Cohort studies recruit volunteers; perhaps there is a form of self-selection occurring such that healthier, more active people would choose to participate in such a study. The effects of the study may be more dramatic in a different sub-population. Due to these design differences, protective effects of specific foods (i.e. apples) or groups of foods (i.e. Brassica vegetables) may have been missed in such studies (Slattery, 2001). In fact, Brassica vegetables have been shown to reduce breast cancer risk. A case-control study of postmenopausal women in Sweden found no relationship between total fruit and vegetable consumption and breast cancer (Terry *et al.*, 2001). They did, however, find a significant protective effect of Brassica vegetables, with a relative risk of 0.58

for the upper decile (who consumed 1.5 servings/day) compared to the lowest decile (virtually no consumption).

A study in 1992 analyzed the effects of various anthropometric, nutritional and hormonal factors on prognostic characteristics of early-detected breast tumors in 91 women. Fruits and vegetables were the only food groups that were significantly associated with a more favorable prognosis, namely more differentiated tumors, less vascular invasion, and positive estrogen and progesterone receptor status (Ingram *et al.*, 1992).

It is unclear whether increased fruit and vegetable intake protects against breast cancer indirectly, by lowering fat and increasing fiber in the diet, or directly, through the bioactivity of the phytochemicals. Some epidemiology studies have attempted to sort out the contribution of different classes of phytochemicals. For example, a cohort study in Finland found that total flavonoids non-significantly reduced breast cancer (R.R. 0.72 for highest quintile vs. lowest) (Knekt *et al.*, 1997) but a later follow-up found quercetin to be significantly associated with reduced risk (R.R. = 0.62) while other flavonoids were not (Knekt *et al.*, 2002). A case-control study in Greece found an inverse association with flavones (O.R. = 0.87) but not other classes of flavonoids (Peterson *et al.*, 2003). A large case-control study in Italy (2,569 cases and 2,588 controls from 1991-1994) found a significant inverse relationship between breast cancer and flavones (O.R. = 0.81) and flavonols (O.R. = 0.80), while 4 other classes of flavonoids had non-significant associations (flavanones O.R. = 0.95; flavan-3-ols O.R. = 0.86; anthocyanidins O.R. = 1.09; isoflavones O.R. = 1.05) (Bosetti *et al.*, 2005).

Slattery (2001) discussed the strengths and limitations that the various types of epidemiology studies (clinical, case-control, and cohort) have in determining the association between diet and cancer. She underscores the importance of timing and



duration of exposure, considering that certain foods such as fruits and vegetables may exert protective effects at a specific stage in the disease process. In that light, animal and cell studies become extremely valuable in helping to uncover the mechanisms by which whole foods or individual phytochemicals can prevent or slow the growth of cancer. That information, in turn, is crucial for effective and careful design of dietary intervention studies.

#### **IV. APPLES AND BREAST CANCER**

**A. Apples.** Phytochemicals are classified according to structure, the largest sub-class being phenolic compounds which includes further sub-classes such as phenolic acids, flavonoids, and tannins (Liu, 2004). Among commonly consumed fruits, apples contain the second highest phenolic content and second highest antioxidant activity (Sun *et al.*, 2002). However, since they are more popular than other fruits, apples are the number one source of phenolics in the American diet (Wolfe *et al.*, 2008).

**1. Phytochemical Composition of Apples.** Our lab has improved the analysis of total phenolic content of fruits and vegetables by accounting for bound phenolics, in addition to the standard free phenolics (Sun *et al.*, 2002). This method was used in our lab to determine the total phenolic content of various fruits. The phenolic content was measured using a colorimetric Folin-Ciocalteu method with gallic acid as a standard. Results were presented as mg of gallic acid equivalents per 100 g of the edible parts of the fruit. Out of the 11 commonly consumed fruits tested, apple had the second highest total phenolic content, while cranberry had the highest. The total phenolic content of the apple in this study was  $296.3 \pm 6.4$  mg/100g, while cranberry contained  $527.2 \pm$

21.5 mg/100 g. However, since apples are consumed far more than cranberries, they remain a significant source of phenolics in the diet (Sun *et al.*, 2002).

Our lab also measured the distribution of phenolics among the flesh and peel of the apple (Wolfe *et al.*, 2003). In 4 apple varieties, the phenolic contents of the flesh, the peel, and the flesh with the peel (whole apples) were measured. In Idared, Rome Beauty, Golden Delicious, and Cortland apples, total phenolic contents of the flesh were  $75.7 \pm 4.0$ ,  $93.0 \pm 4.1$ ,  $97.7 \pm 8.9$ , and  $103.2 \pm 12.3$  mg/100 g fruit, respectively. In the same order, total phenolic contents of the flesh with the peel were  $120.1 \pm 15.0$ ,  $159.0 \pm 15.1$ ,  $129.7 \pm 9.7$ , and  $119.0 \pm 14.9$  mg/100 g fruit. Total phenolic contents of the peel were  $588.9 \pm 83.2$ ,  $500.2 \pm 13.7$ ,  $309.1 \pm 32.1$ , and  $388.5 \pm 82.4$  mg/100 g fruit. The interesting trends here are the similar levels of phenolics in the flesh and the flesh with peel among the four varieties, while the amounts in the peel varied widely among the varieties. Most notable is the fact that the phenolics are highly concentrated in the peel (Wolfe *et al.*, 2003).

In a study of 8 apple varieties, five major groups of polyphenolic compounds were detected by HPLC: flavan-3-ols, flavonols, dihydrochalcones, anthocyanins, and phenolic acids (hydroxybenzoic acid and hydroxycinnamic acids) (Tsao, *et al.* 2003). The major group was the flavan-3-ols, or catechins, which can account for 55.7% of polyphenols in the flesh (0 to 583.0  $\mu\text{g/g}$ ) and 59.7% of polyphenols in the peel (151.3 to 1,654.8  $\mu\text{g/g}$ ). The dominant catechins in apples are catechin and epicatechin (stereoisomers), and the dimers procyanidin B1 (epicatechin plus catechin) and procyanidin B2 (2 epicatechin molecules) (Tsao *et al.*, 2003).

The second major group of polyphenols in apples was the flavonols, such as quercetin, kaempferol and myricetin. Quercetin and its glycosides are by far the most abundant flavonols, can contribute up to 18% of total phenolics, and occur exclusively

in the peel. Concentrations were found to be 220.3 to 349.9  $\mu\text{g/g}$  fresh weight (Tsao *et al.*, 2003).

The other three minor polyphenolic groups found in these 8 apple varieties were dihydrochalcones, anthocyanins, and phenolic acids. The main dihydrochalcones were phloretin 2'-glycoside (phlorizdin) and phloretin 2'-xyloglucoside, which occurred mostly in the peel in average concentrations of 124  $\mu\text{g/g}$  fresh weight. Anthocyanins, which occur as cyanidin glycosides in red apples, ranged from 42.9 to 208.2  $\mu\text{g/g}$  fresh weight. The two main groups of phenolic acids found were hydroxybenzoic acids and hydroxycinnamic acids. Hydroxybenzoic acids can account for less than 5% of phenolics in apples, at concentrations so low they may often be below detection levels. In the peel, total benzoic acids may be between 40 and 80  $\mu\text{g/g}$  fresh weight. The major hydroxycinnamic acid found in the apples was chlorogenic acid, in concentrations of 136  $\mu\text{g/g}$  fresh weight in the peel and 177  $\mu\text{g/g}$  fresh weight in the flesh. Two other hydroxycinnamic acids, p-coumaric acid and caffeic acid, occur in apples in lower concentrations (Tsao *et al.*, 2003).

In our lab, 29 compounds were isolated from red delicious apple peels, including triterpenoids, flavonoids, organic acids, and plant sterols (He and Liu, 2007 and 2008). Extracts of apple peel were fractionated by extraction with 80% acetone, followed by subsequent extractions with ethyl acetate and water-saturated n-butanol. Ethyl acetate fractions were subject to silica gel chromatography while butanol fractions were subject to dianion HP-20 column chromatography, followed by silica gel chromatography. Individual compounds were collected and further separations were achieved by HPLC. Quercetin-3-O- $\beta$ -D-glucopyranoside comprised 82.6% of the total flavonoids in the peel, while quercetin-3-O- $\beta$ -D-galactopyranoside comprised 17.1%. Trace amounts of quercetin (0.2%) were detected along with (-)-catechin, (-)-

epicatechin, and quercetin-3-O- $\alpha$ -L-arabinofuranoside. In this study, two new compounds were isolated from apple peel and identified for the first time: 3 $\beta$ -trans-p-coumaroyloxy-2 $\alpha$ ,3 $\beta$ ,13 $\beta$ -trihydroxy-urs-11-en-28-oic acid, and its cis counterpart, 3 $\beta$ -cis-p-coumaroyloxy-2 $\alpha$ ,3 $\beta$ ,13 $\beta$ -trihydroxy-urs-11-en-28-oic acid (He and Liu, 2008).

Thirteen triterpenoids were also isolated from the peels of red delicious apples (He and Liu, 2007). Four of the triterpenoids were isolated and identified for the first time: Compound 3, identified as 3 $\beta$ -trans-cinnamoyloxy-2 $\alpha$ -hydroxyurs-12-en-28-oic acid; Compound 7, identified as 2 $\alpha$ -hydroxy-3 $\beta$ -{[(2E)-3-phenyl-1-oxo-2-propenyl]oxy}olean-12-en-28-oic acid; Compound 8 (an isomer of compound 7), 2 $\alpha$ -hydroxy-3 $\beta$ -{[(2Z)-3-phenyl-1-oxo-2-propenyl]oxy}olean-12-en-28-oic acid; and Compound 12, 2 $\alpha$ ,3 $\beta$ ,13 $\beta$ -trihydroxyurs-11-en-28-oic acid. Compound 1, ursolic acid, was the most abundant triterpenoid in the apple peel. Compound 2 was identified as 2 $\alpha$ -hydroxyursolic acid, and Compound 9 as 3 $\beta$ -trans-p-coumaroyloxy-2 $\alpha$ -hydroxyolean-12-en-28-oic acid. Four of these compounds (2 $\alpha$ -hydroxyursolic acid, 3 $\beta$ -trans-cinnamoyloxy-2 $\alpha$ -hydroxyurs-12-en-28-oic acid, 2 $\alpha$ -hydroxy-3 $\beta$ -{[(2E)-3-phenyl-1-oxo-2-propenyl]oxy}olean-12-en-28-oic acid and 3 $\beta$ -trans-p-coumaroyloxy-2 $\alpha$ -hydroxyolean-12-en-28-oic acid) potently inhibited MCF-7 cell proliferation. This will be discussed further in a later section (He and Liu, 2007).

**2. Metabolism and Bioavailability.** Metabolism and bioavailability are critical factors in determining the beneficial health effects of dietary phytochemicals: How are the compounds metabolized by the digestive system? Are the compounds readily absorbed into circulation and available to tissues? Many cell, animal, and human feeding studies have contributed to answering these questions. For the purposes of this review, we will focus on quercetin and its glycosides.

The metabolism and absorption of flavonoids was summarized nicely by Williamson *et al.* (2000). Briefly, quercetin aglycone may passively diffuse from the lumen into the epithelial cells of the small intestine due to its hydrophobic nature, and enter circulation. Quercetin may be hydroxylated, methylated, sulphated, or glucuronidated in the small intestine, liver, or the kidney. Methylation and demethylation may occur at the 3' and 4' positions, resulting in the metabolite 3'-methylquercetin being detected in human plasma (Manach *et al.*, 1998). Sulphation and glucuronidation are the major pathways of quercetin metabolism, with sulphation first becoming saturated at lower concentrations (Morand *et al.*, 1998; Piskula and Terao, 1998). When fed either quercetin-3-glucoside or quercetin-4'-glucoside, quercetin glucuronides were found in human plasma, not quercetin glycosides (Sesink *et al.*, 2001). Sulphated or glucuronidated quercetin conjugates may also be methylated on their catechol groups, as seen in bile of rats perfused with quercetin (Crespy *et al.*, 2003). Sulphates of quercetin will be excreted in the urine, while the liver will release the quercetin glucuronides with the bile back into the small intestine (Mulder and Jakoby, 1990). Glucuronides continue on to the large intestine where microbes hydrolyze them and free the aglycone to be reabsorbed. In this way, phytochemicals may be caught up in enterohepatic circulation, increasing the elimination half-life in the body (Williamson *et al.*, 2000). Microbes in the colon may hydrolyze glycosides, conjugates, and also degrade the phenolics themselves. Alternatively, rather than diffusing into the circulation from the small intestine, quercetin may pass through the lumen of the small intestine directly into the colon and be subject to degradation by microbes (Hackett, 1986).

Quercetin glycosides follow a slightly different path. Being hydrophilic, glycosides do not freely diffuse across membranes. Glycosides may be hydrolyzed off

of the quercetin in the lumen of the small intestine by lactase phlorizin hydrolase (LPH), a type of  $\beta$ -glycosidase found in the brush border of the small intestine (Walle *et al.*, 2000; Day *et al.*, 2003). Then, quercetin aglycone may passively diffuse and be metabolized as described above. Alternatively, quercetin glycosides may be actively transported into the small intestinal cells by active glucose transporters such as SGLT1 (sodium-dependent glucose transporter 1) (Lostao *et al.*, 1994; Gee *et al.*, 1998) or facilitative-type glucose transporters such as GLUT2 (Kellett, 2001), and then cleaved by intracellular  $\beta$ -glycosidases. Again, the quercetin aglycone is further metabolized as described above.

Quercetin glycosides are metabolized differently depending on the sugar moiety. For example, as summarized by Williamson *et al.* (2000), cytosolic  $\beta$ -glucosidase from human small intestine and liver cells had high affinity for quercetin-4'-glucoside but exhibited no hydrolyzing activity on quercetin-3-rutinoside (Day *et al.*, 1998). Rat intestinal  $\beta$ -glucosidase had higher activity on quercetin-4'-glucoside than on quercetin-3-rutinoside (Ioku *et al.*, 1998). Cytosolic  $\beta$ -glucosidase from human liver hydrolyzed quercetin-4'-glucoside but not quercetin-3-glucoside. The human and rat small intestine enzyme, lactase phlorizin hydrolase (LPH, a different kind of  $\beta$ -glycosidase) hydrolyzed quercetin-3-glucoside (Leese and Semanza, 1973). Walgren *et al.* (2000) used a Caco-2 cell model to show for the first time that quercetin-4'-glucoside was absorbed into the cells via the SGLT1 glucose transporter (Walgren *et al.*, 2000).

Wolffram *et al.* (2002) investigated the absorption of Q-3-G from the mucosal side of a rat jejunum mounted in Ussing-type chambers. Under control experimental conditions, only 14% of the Q-3-G remained on the mucosal side. Upon addition of phloridzin (a competitive inhibitor of SGLT1) and sodium-free medium, 54% of the Q-3-G remained. To test whether the GLUT5 transporter was involved, D-fructose

was added as a competitive inhibitor, which had no effect on disappearance of Q-3-G from the mucosal side. The same experiments were repeated with sections of proximal colon, which do not express SGLT1; no Q-3-G was taken up by the tissue. The authors conclude SGLT1 transport is a major contributing means of Q-3-G uptake by small intestinal cells, and summarize the various possible pathways, all of which may play a role. Q-3-G may be cleaved by LPH in the brush border (Day *et al.*, 2000) then released as an aglycone into the intestinal lumen; or Q-3-G may be taken up into the intestinal cell, cleaved by intracellular enzymes, and then the aglycone may diffuse back into the lumen or through to the serosal side. Uptake into the cell may be by SGLT1 transporters, but not GLUT 5 transporters. GLUT2 transporters may contribute as other studies have indicated (Kellett, 2001).

Arts *et al.* (2002) contested Wolffram's findings, pointing out that both glucose and phloridzin, used as inhibitors of SGLT1 by Wolffram, also have been shown to inhibit LPH. Arts *et al.* (2002) also deem the presence of quercetin aglycone on the mucosal side and the absence of conjugates as further evidence of the action of LPH. Wolffram of course responded to these criticisms, highlighting the absence of sodium in their model as a means of inactivating the SGLT1 transporter, while LPH is not sodium dependent (Wolffram, 2002). In addition, Wolffram points out that free aglycone from LPH hydrolysis should be subject to the same conjugation as any taken up by SGLT1 and subsequently hydrolyzed by intracellular glycosidases. They therefore maintain that lack of conjugates in their model does not disprove SGLT1 transport (Wolffram, 2002).

Day *et al.* (2003) found that quercetin-3-glucoside was hydrolyzed solely in the small intestinal lumen by LPH and not by cytosolic  $\beta$ -glucosidase, and that Q-3-G was not transported by SGLT1, while quercetin-4-glucoside used both LPH and SGLT1. This group used rat everted-jejunal sacs as a model, and utilized phloridzin as

a SGLT1 inhibitor, and N-(n-butyl)-deoxy-galactonojirimycin (NB-DGJ) as an LPH inhibitor.

Finally, in a study published in 2003, Sesink *et al.* (2003) conceded that transport by SGLT1, as proposed by Wolffram (2002), may play a role in Q-3-G absorption. Sesink *et al.* (2003) performed an *in situ* perfusion of rat small intestine with Q-3-G in solution. In the control, 29% of the Q-3-G was absorbed out of the perfusion fluid. Addition of NB-DGJ in doses of 0.5, 2 and 10 mmol/L inhibited hydrolysis of the Q-3-G by 38, 50 and 67%, respectively, confirming the significant role of LPH in intraluminal hydrolysis of Q-3-G. Sesink recalls that the absence of sodium in Wolffram's model inhibited Q-3-G disappearance from the mucosal medium by 25% (Wolffram *et al.*, 2002), noting the similarity to the extent of Q-3-G absorption that was unaffected by LPH inhibition in their own study. Therefore, Sesink *et al.* concluded that transport of Q-3-G by SGLT1 transporter could not be completely discounted (Sesink *et al.*, 2003).

While each of the mentioned studies contributed important information regarding the absorption and metabolism of quercetin and its glycosides, inconsistencies occur between studies and many questions remain. Differences in experimental design may account for varying results and interpretations. Such differences include: the model used (Caco-2 cell culture, rat intestinal tissues, rat or human *in situ* perfusion); duration of exposure (15 or 30 min., 1-2 h); test compound (quercetin aglycone or various glycosides); concentrations of test compounds; which compounds and metabolites were measured (such as aglycone, glycosides, and methylated, sulfated and glucuronidated conjugated; these papers used HPLC); and use of proper inhibitors for the enzymes and glucose transporters of interest.

According to Boyer and Liu (2004), quercetin glycosides found in apples have varied bioavailabilities. Quercetin-3-glucoside is readily hydrolyzed by LPH as



mentioned and absorbed in the small intestine, while other glycosides of quercetin such as rhamnosides, xylosides, and galactosides are not as easily absorbed. These compounds would then likely continue on to the colon and be subject to hydrolysis and further degradation by intestinal microflora. Metabolism of other prominent phytochemicals found in apples such as phloridzin, chlorogenic acid, catechin and epicatechin, is described by Boyer and Liu (2004).

Under typical consumption of plant foods, the blood concentrations of many polyphenols was estimated to range from 1 to 10  $\mu\text{M}$  (Wang *et al.*, 2003). In the Netherlands, flavonol consumption was measured at about 23 mg/d (Aziz *et al.*, 1998). A broad range of flavonol and flavone intakes was recorded by the Seven Countries Study, which found the Finnish cohort to consume on average 3 mg/d while the Japanese cohort consumed 70 mg/d (Hertog *et al.*, 1995).

Bioavailability of phytochemicals, rate of absorption, peak plasma concentrations and elimination half-lives depend on the type of sugar moiety and the food matrix. As summarized by Ross and Kasum (Ross and Kasum, 2002), many studies have observed absorption of phytochemicals from various foods. Hollman *et al.* (1995) restricted ileostomy patients from all quercetin for 12 days, then the patients consumed a source of quercetin for 12 days. Patients who ate fried onions (which contain quercetin glucosides) absorbed 52% of the quercetin, while those who consumed pure quercetin aglycone absorbed only 24%. Another study by Hollman *et al.* (1997) demonstrated that in healthy subjects, quercetin from a single dose of apple or pure quercetin-3-rutinoside was absorbed only about 30% compared to that absorbed from onions. Apple quercetin reached peak plasma levels after 2.5 hours with an elimination half-life of 23 hours, while onion quercetin peaked after only 0.7 hours, with a longer half-life of 28 hours. DeVries *et al.* (1998) also demonstrated the

high bioavailability of quercetin found in onions: in healthy subjects, the amount of quercetin absorbed from black tea was only half that absorbed from the onions.

Olthof *et al.* (2000) fed 9 subjects a single dose of 325  $\mu$ M Q-3-G, equivalent to 150.9 mg, and found the resulting plasma concentration of quercetin to peak at  $5.0 \pm 1.0$   $\mu$ M after only  $37 \pm 12$  minutes. A single dose of 331  $\mu$ M quercetin-4'-glucoside caused a peak plasma concentration of  $4.5 \pm 0.7$   $\mu$ M quercetin by  $27 \pm 5$  minutes, with an elimination half-life of  $17.7 \pm 0.9$  hours. Since the absorption of Q-3-G and Q-4'-G are nearly equivalent, the following study on quercetin-4'-glucoside metabolism may prove useful in the discussion of Q-3-G as well.

Graf *et al.* (2005) investigated the absorption, metabolism and disposition of quercetin-4'-glucoside (Q-4'-G), a major flavonol found in onions. (2-<sup>14</sup>C)Q-4'-G was synthesized and fed to rats (7.6 mg/kg), and plasma and tissues were harvested at 0.5, 1, 2, and 5 hours post-ingestion. Q-4'-G was detected only in the gastrointestinal (GI) tract at 0.5 and 1 h, with a small amount in the liver at 0.5 h, and none at all in the kidneys and plasma. Quercetin aglycone accounted for 25% of the radioactivity in the GI tract at 0.5 h, which reduced to < 8% at later time points. Very small amounts of quercetin were found in the liver and kidney, but none in the plasma. Methylated and unmethylated glucuronides of quercetin were present in all organs tested at 0.5, 1 and 2 h. At 5 h, only a small amount remained in the liver. Methylated and unmethylated glucuronides were the major group of metabolites in the liver and kidney at all timepoints, in the plasma at 0.5 h, and in the GI tract at 5 h. Sulfates of quercetin may also occur methylated and/or glucuronidated. Glucuronidated sulfates increased over time in the plasma, accounting for 76% of the metabolites in plasma at 5 h. Quercetin sulfate and methyl-quercetin sulfate were found only in the intestine at 0.5, 1 and 2 h (Graf *et al.*, 2005).

Graf *et al.* (2005) conclude only 6% of the dose of Q-4'-G was absorbed into the blood and internal organs, citing the consistency with human feeding studies of onions or Q-4'-G supplements which resulted in 1.4 to 6.4% of the ingested dose of quercetin being detected in the urine (Hollman *et al.*, 1997; Olthof, 2000; Graefe *et al.*, 2001). The authors estimated the bioavailability of Q-4'-G from the diet or from supplements to be between 1 and 7% (Graf *et al.*, 2005).

The authors cite other studies which may have dramatically over-estimated the absorption of quercetin. For example, studies on ileostomy patients calculated > 50% of the dose was absorbed from the GI tract (Hollman *et al.*, 1995; Walle *et al.*, 2000). Graf *et al.* (2005) point out these researchers measured quercetin, quercetin glycosides and quercetin conjugates, but not methyl-quercetin. Approximately 44% of quercetin becomes methylated and therefore if that methyl-quercetin were undetected, it may have been mistakenly assumed that portion of the quercetin sample had been absorbed out of the intestine (Graf *et al.*, 2005).

Another example is a study by Wall *et al.*, (2001), in which human subjects were fed a radio-labeled dose of quercetin aglycone. Based on radioactivity measurements of plasma over 72 h, the researchers concluded 36-53% of the dose was absorbed. However, based on previous studies showing plasma quercetin returns to baseline within 24 h, Graf suspects the radioactivity in plasma over the 72 h study was CO<sub>2</sub> from microbial degradation of the quercetin, en route to lungs for exhalation. Graf considers the urinary excretion of quercetin, 4.2% of the dose, as the more reliable indicator of absorption (Graf *et al.*, 2005).

Since the entire dose of Q-4'-G in the rat study was metabolized within 2 h, and at all time points 86-93% of the dose was in the GI tract (while no more than 2% was in liver at any time point), Graf *et al.* conclude most of the transformation occurs in the GI tract. This is consistent with an *in situ* rat perfusion study of quercetin, where

90% of the quercetin conjugates were found in the intestinal eluent, but only 10% in the bile. At the first timepoint, 0.5 h, Graf *et al.* saw more metabolites in the liver, kidneys and plasma than the GI tract, concluding the Q-4'-G was metabolized in the intestine, absorbed, and further methylated and glucuronidated in the liver and kidneys (Graf *et al.*, 2005).

In summary, quercetin, whether the aglycone or a glycoside, will be absorbed to a certain extent. The quercetin conjugates found in the circulation ultimately are excreted in the urine or the bile. Quercetin that reaches the colon either directly from the small intestine or from the bile may be degraded further by intestinal microflora. Elimination half-lives differ somewhat according to the form of quercetin and the food source, and so regular consumption to maintain plasma levels is recommended to achieve the maximum health benefits.

**B. Epidemiology Studies Linking Apple Consumption to Reduced Breast Cancer Risk.** A recent epidemiologic study including 2,569 breast cancer patients showed that apple consumption was linked to a lower risk of breast cancer (Gallus *et al.*, 2005). When compared with subjects consuming <1 apple/day, the multivariate odds ratios (OR) for  $\geq 1$  apple/day were 0.82 (95% CI 0.73-0.92) for breast cancer. After further allowance for consumption of vegetables and other fruits, the association with apples became even stronger for breast cancer (OR 0.76, CI 0.67-0.85) (Gallus *et al.*, 2005). A case-control study in Brazil investigated associations between breast cancer occurrence and various anthropometric, socio-economic, and dietary factors (DiPietro *et al.*, 2007). Thirty-three women recently diagnosed with breast cancer comprised the case group, while 33 healthy volunteers formed the control group. Breast cancer incidence was strongly correlated with age ( $\geq 45$  y, OR = 4.00), low family income (OR = 3.50), low educational level (primary school or less, OR = 16.7), and menopause (OR = 9.84). Adjusting for age changed the odds ratios for low family

income to 4.51 and for low education level to 11.02. Since income is associated with dietary habits, the authors adjusted for family income before analyzing the dietary associations. They then found that breast cancer was increased by consumption of pig lard (OR = 6.32) and fatty red meat (OR = 3.48), while consumption of apples (OR = 0.30), watermelons (OR = 0.31) and tomatoes (OR = 0.16) reduced risk (DiPietro *et al.*, 2007).

**C. Animal Studies of Effects of Apples on Mammary Cancer.** Our lab has shown that apples fed to Sprague-Dawley rats in doses equivalent to human consumption of 1, 3, or 6 apples per day significantly reduced DMBA-induced mammary tumor development (Liu *et al.*, 2005). Rats were fed whole apple extracts beginning 2 weeks prior to DMBA administration, and continuing throughout the 24 week study. In a clear dose-dependent manner, the apple extracts down-regulated PCNA, Cyclin D1 and Bcl-2, and up-regulated Bax and nuclear fragments, reduced tumor size and tumor burden, and delayed tumor onset (Liu *et al.*, 2009). The experimental design of beginning the apple feeding prior to DMBA administration, as well as the effects on early markers such as PCNA and Cyclin D1, indicate the apple interfered with the initiation and promotion stages of carcinogenesis. Since the apple feeding continued throughout the duration of the experiment, it is unclear whether the treatment also affected progression.

**D. Cell and Molecular Studies.** The mechanisms of action by which fruits and vegetables exert their health benefits are not completely understood. However, cell culture models and molecular techniques are valuable and efficient tools for assessing the various anti-cancer functions of individual phytochemicals. The mechanism of action of phytochemicals that has gained much attention recently is antioxidant activity, which is a measure of how effectively the compounds can quench free radicals. In doing so, the phytochemicals reduce oxidative stress (the imbalance

between oxidants and antioxidants) which is associated with chronic diseases such as cancer since the free radicals can damage DNA, proteins, and lipids (Ames and Gold, 1991; Ames *et al.*, 1993; Liu and Hotchkiss, 1995). Cells under oxidative stress are susceptible to DNA damage, which may lead to mutations that alter expression or activity of key regulatory proteins. Such mutations can result in de-regulation of cell cycle and subsequent uncontrolled cell proliferation, which can lead to cancer. These cells are then unable to properly respond to further oxidative stress and are highly susceptible to additional DNA damage. This cycle contributes to increasing genetic instability characteristic of tumor cells (Bartek and Lukas, 2001).

While phytochemicals can protect DNA from oxidative stress, they can also help prevent cancer by modifying cell cycle progression or inducing apoptosis in the event DNA damage does occur. Phytochemicals also regulate gene expression (genes governing cell proliferation and differentiation, oncogenes and tumor suppressor genes), enzyme activity (detoxification, oxidation and reduction enzymes), immune system function and hormone metabolism (Liu, 2004).

**1. Whole Apple Extracts.** Whole apple extracts and apple peels have been shown to have potent anti-oxidant activity (Eberhardt *et al.*, 2000; Wolfe *et al.*, 2003; Wolfe and Liu, 2007). Eberhardt *et al.* (2000) found that the Vitamin C in an apple accounts for only 0.32% of the fruit's total antioxidant capacity, implicating the other phenolic compounds in the remaining 99.68% of the activity. Using the TOSC (total oxyradical scavenging capacity) assay, our lab determined the antioxidant activity of whole apple extract was  $97.6 \pm 4.6$   $\mu\text{mol}$  of vitamin C equivalents/g of fresh weight of the fruit (Sun, et al. 2002). Out of the 11 commonly consumed fruits analyzed in that study, the antioxidant activity of apple was second only to cranberry, which was  $177.0 \pm 4.3$   $\mu\text{mol/g}$ . Later, antioxidant activity in the flesh of the apple was compared to that

in the peel (Wolfe *et al.*, 2003). In Idared, Rome Beauty, Golden Delicious, and Cortland apples, antioxidant activity of the flesh was  $46.9 \pm 1.6$ ,  $68.02 \pm 1.47$ ,  $43.5 \pm 0.4$ , and  $50.4 \pm 2.2$   $\mu\text{mol Vit. C eq/g fruit}$ , respectively. In the same order, antioxidant activity of the flesh with the peel was  $72.2 \pm 2.3$ ,  $131.6 \pm 0.8$ ,  $66.9 \pm 1.8$ , and  $83.5 \pm 2.3$   $\mu\text{mol Vit. C eq/g fruit}$ . Antioxidant activity of the peel was  $312.2 \pm 9.8$ ,  $228.4 \pm 6.7$ ,  $111.4 \pm 4.7$ , and  $159.0 \pm 4.3$   $\mu\text{mol Vit. C eq/g fruit}$ . Similar to the total phenolic content, antioxidant activity was higher in the peel than the flesh (Wolfe *et al.*, 2003).

Our lab has shown that non-cytotoxic doses of whole apple extract inhibit proliferation of MCF-7 cells (Yoon and Liu, 2007; Sun and Liu, 2008; Felice *et al.*, 2009). Apple extracts effectively inhibited TNF- $\alpha$ -induced NF- $\kappa$ B activation, which is a significant finding since NF- $\kappa$ B plays a role in resistance to chemotherapy in cancer cells (Yoon and Liu, 2007). It is important to note that the anti-proliferative activity of whole apple extracts is not due to phenolic-induced  $\text{H}_2\text{O}_2$  formation in the culture medium (Liu and Sun, 2003), as was previously suspected.

## **2. Pure Phytochemicals.**

**a. Quercetin.** Quercetin isolated directly from fresh red delicious apple peels in our lab exhibited anti-proliferative activity against MCF-7 cells *in vitro* with an  $\text{EC}_{50}$  of  $137.5 \pm 2.6$   $\mu\text{M}$ , while the quercetin-3-O- $\beta$ -D-glucopyranoside was even more potent with an  $\text{EC}_{50}$  of  $23.9 \pm 3.9$   $\mu\text{M}$  (He and Liu, 2008).

MDA-MB468 cells, an estrogen receptor negative human breast cancer cell line, express a mutated form of p53 that is required for their survival. Avila *et al.* (1994) found that quercetin effectively reduced proliferation of these cells in a dose-dependent manner after only 24 hours, with an  $\text{EC}_{50}$  of 7.0  $\mu\text{g/mL}$  after a 72 hour exposure. Cell cycle analysis first showed a G2/M accumulation; when the cells were synchronized first, a G1 accumulation occurred. Together these results indicate the target of quercetin is present throughout the cell cycle. Quercetin down-regulated

mutant p53 expression in a time- and dose-dependent manner (2 through 8 hours, 10 – 75 µg/mL). The authors verified the quercetin was not causing an overall inhibition of protein synthesis within the cell, and that this effect was specific to the mutant p53. mRNA levels remained stable with quercetin treatment, and labeling with [<sup>35</sup>S]methionine with or without quercetin revealed the phytochemical was preventing protein synthesis of the p53 (Avila *et al.*, 1994). Interestingly, two quercetin glycosides (quercetrin and quercetin-3-rutinoside) did not affect p53 levels. Quercetin appeared to have a specificity for the critical mutant p53 protein in the MDA-MB468 cell line (Avila *et al.*, 1994).

In MCF-7 cells, quercetin was shown to induce a G2-phase accumulation with an accompanying increase in Cyclin B1 expression and Cdc2 kinase activity after more than 24 hours treatment (Choi *et al.*, 2001). Increased p21 expression and association with Cdc2/Cyclin B1 was seen after 48 hours of treatment, as was a p21-dependent apoptosis induction. Quercetin induced a G1 cell cycle arrest and apoptosis in MDA-MB-453 mammary carcinoma cells and down-regulated Bcl-2 expression while up-regulating Bax expression and cleavage of caspase-3 and PARP (Choi *et al.*, 2008).

Her-2/*neu*, a receptor tyrosine kinase (RTK) that functions through dimerizing with other RTKs, is an important player in breast cancer signaling (Olayioye *et al.*, 2000; Hynes and Lane, 2005). Quercetin caused down-regulation of Her-2/*neu* receptor, along with corresponding decreases in phosphorylation of PI3K and Akt, in human breast carcinoma SK-Br3 cells which over-express Her-2/*neu* (Jeong *et al.*, 2008). The investigators saw a similar down-regulation of the Her-2/*neu* receptor by quercetin in MCF-7 cells, and in a Her-2/*neu* over-expressing ovarian cancer cell line SK-Ov3.



Quercetin has been shown to interfere with signaling pathways involved in migration and metastasis. Matrix metalloproteinases are responsible for dissolving the extracellular matrix, allowing tumor cells to migrate and invade distant sites (Duffy *et al.*, 2000). In MCF-7 cells, 50 ng/mL TPA (12-O-tetradecanoylphorbol-13-acetate) induced invasion, colony formation, and migration as determined by an *in vitro* invasion assay, a soft agar assay, and an *in vitro* monolayer wound-healing assay, respectively (Lin *et al.*, 2008). 80  $\mu$ M Quercetin aglycone (but not quercetin-3-O-rhamnoside or quercetin-3-O-rutinoside) was able to reverse these effects of TPA. Furthermore, quercetin inhibited enzyme activity of MMP-9 by down-regulating expression of both the mRNA and the protein. In this cell model, activation of PKC $\delta$  was necessary for the TPA-induced MMP-9 activation and subsequent cell migration. TPA activated ERK and JNK but not p38 MAPK or Akt. Through the use of inhibitors the authors determined that AP-1, not NF- $\kappa$ B (both transcriptional regulators of MMP-9), was involved in the TPA-induced activation of MMP-9. TPA-treated cells showed up-regulation of c-Jun but not c-fos. Quercetin was systematically shown to block TPA-induced PKC $\delta$  activation, ERK and JNK activation, c-Jun expression, and AP-1 promoter activity. Structure-activity-relationship experiments determined that the hydroxyl (OH) groups on C3' and C4' confer quercetin's ability to suppress MMP-9 expression, while additional OH groups on C3, C5 or C7 would enhance that ability (Lin *et al.*, 2008).

In a two-day cell proliferation assay, quercetin inhibited proliferation in a dose-dependent manner in three mammary cell lines: normal Human Mammary Epithelial Cells (HMEC), spontaneously immortalized human breast epithelial cells (MCF-10A), and ER+ breast cancer cells (MCF-7). The quercetin was most effective against the HMECs, with an IC<sub>50</sub> of 5  $\mu$ g/mL quercetin (14.8  $\mu$ M). The IC<sub>50</sub> against MCF-7 cells was 13  $\mu$ g/mL quercetin (38.4  $\mu$ M), while the MCF-10A cells were the

most resistance with an IC<sub>50</sub> of 32µg/mL quercetin (94.6 µM) (Hakimuddin *et al.*, 2004).

The effects of physiologically relevant doses of quercetin (1, 5 and 10 µM) were investigated in 3 human breast carcinoma cell lines, SK-BR3, MDA-MB-453, and MDA-MB-231, and one normal breast cell line, MCF-10A (Jeong *et al.*, 2009). Over 4 days of treatment, quercetin inhibited proliferation in a dose-dependent manner in SK-Br3 and MDA-MB-453 cells, but had no effect on proliferation of MCF-10A cells. After 4 days, viability of SK-Br3 cells was >95% and there was no evidence of PARP cleavage, which was used as a marker of apoptosis. Rather, the quercetin was causing a cell cycle block at the G1 to S transition. The proportion of SK-Br3 cells in G0/G1 phase increased from 61.8% in the control to 75.9% in those treated with 10 µM quercetin; S phase cells decreased from 30.2% in control to 18.2% with 10 µM quercetin; and G2/M phase cells did not change significantly. Over 2 and 4 days of treatment, quercetin induced hypo-phosphorylation of Rb (Ser<sup>780</sup> and Ser<sup>807/811</sup>) in SK-Br3 cells. Additionally, quercetin induced DNA damage, and subsequent phosphorylation of histone H2A.X, Chk2 activation, and p21 induction. In these cells p53 expression was not affected, nor was expression of Cyclin D1, CDK4, CDK6, Cyclin E or CDK2. However, Cyclin B and CDK1 were down-regulated. Quercetin also induced p21 expression in MDA-MB-453 cells. In the normal MCF-10A cells, quercetin did not affect cell proliferation over 4 days. After 2 and 3 days of treatment, quercetin had no effect on phosphorylation of Rb, there was no detectable p21 expression, and there were no changes in Cyclin B and CDK1 expression. In this study of physiologically relevant doses, quercetin had clear cell-type specific effects on breast cancer cells *in vitro* which included inhibition of cell proliferation, an accumulation of cells in the G1 phase of the cell cycle, and a p53-independent/Chk2-dependent induction of p21 in SK-Br3 cells, while none of these effects were seen in

normal MCF-10A cells (Jeong *et al.*, 2009). Looking at these two studies together (Hakimuddin *et al.*, 2004; Jeong *et al.*, 2009), it is clear that quercetin exerts its anti-cancer effects in both a cell type- and a dose-specific manner. Further *in vitro* studies in normal and cancerous cell lines, as well as *in vivo* studies, are needed to clarify the mechanisms of action by which quercetin protects against breast cancer.

**b. Flavanols (Catechins).** A class of polyphenols called flavanols or catechins is found in green tea and chocolate, among other food sources (Goodin *et al.*, 2002). Being the major flavanol in green tea, EGCG has been extensively studied. Catechin and epicatechin, however, the main flavanols in apple, are less prevalent in the literature.

Catechins have been found to inhibit breast cancer cells *in vivo* (Morre *et al.*, 2000), and epidemiology studies have demonstrated an inverse relationship between green tea consumption and breast cancer development and recurrence (Nakachi *et al.*, 1998). The authors correlated breast cancer diagnosis and prognostic factors with prior green tea consumption in 472 patients. In premenopausal women diagnosed with Stage I or II breast cancer, increased tea consumption was associated with a decrease in axillary lymph node metastases. In postmenopausal women, the tea was associated with increased expression of both the progesterone and estrogen receptors. A 7-year follow-up study showed that Stage I or II patients who drank  $\leq 4$  cups of green tea per day had a recurrence rate of 24.3%, while those who drank  $\geq 5$  cups per day had a 16.7% recurrence rate. The green tea had no effect on recurrence rates in women diagnosed with a Stage III tumor. This study demonstrates that while green tea is able to affect clinical characteristics of breast cancer, timing of exposure is also important. Goodin *et al.* (2002) investigated the relationship between three common catechins (EGC: epigallocatechin; ECG: epicatechin gallate; EGCG: epigallocatechin gallate)

and estrogen receptor function. *In vitro* assays included receptor binding and ER reporter gene assays in MCF-7 cells. *In vivo*, two markers of estrogen function in immature 57BL/6 mice were measured: uterine weight and uterine peroxidase activity (Goodin *et al.*, 2002). EGC did not have any significant effect in any of the assays, which the authors attribute to the absence of the gallate moiety. EGCG and ECG, however, competed with estradiol ( $E_2$ ) for  $ER\alpha$  and  $ER\beta$  binding in MCF-7 cells, although the affinity of these catechins was nearly 100 to 17,000 times weaker than that of “classical phytoestrogens” (genistein, daidzein and coumestrol). EGCG and ECG did not affect  $E_2$ -induced  $ER\alpha$ -mediated gene expression *in vitro*. However, low concentrations of catechins (0.5  $\mu$ M EGCG co-administered with 0.1 nM  $E_2$ ) increased  $ER\beta$  gene expression by 200%, while high doses (1-50  $\mu$ M EGCG with 1 nM  $E_2$ ) decreased  $ER\beta$  gene expression by 35 – 50% when compared to  $E_2$  control. Catechins alone did not alter the *in vivo* markers of uterine weight or uterine peroxidase formation. Yet, when co-administered with  $E_2$ , EGCG or ECG increased uterine weight significantly and uterine peroxidase formation was ~2.3 times greater than  $E_2$  alone (Goodin *et al.*, 2002). The cancer-protective effect of catechins, therefore, may not be attributed to antagonism of ER function. Gallate-containing catechins (EGCG and ECG) are able to suppress several cytochrome P450s including CYP1A1/2 and CYP 3A4, which are responsible for metabolizing  $E_2$ . The catechins also may influence  $E_2$  uptake into the cells. Taken together, these two activities may contribute to EGCG’s and ECG’s enhancement of  $E_2$  function rather than suppression (Goodin *et al.*, 2002).

In MCF-7 cells, epigallocatechin-3-gallate (EGCG) reduced cell proliferation and induced apoptosis via Akt-dependent survivin suppression (Tang *et al.*, 2007). EGCG is a desirable anti-cancer compound in that it has antineoplastic activity but is

non-cytotoxic to normal cells. Survivin, which suppresses apoptosis by interfering with caspase activity, is expressed in fetal tissues and cancers but expressed at very low levels or not at all in normal adult tissues. Tang *et al.* found the connection between these two observations. In a dose range between 10 and 50  $\mu\text{M}$ , EGCG reduced colony formation and viability (24 hours) and induced apoptosis (Annexin-V staining) in MCF-7 cells. Promoter activity, mRNA and protein expression of survivin were suppressed, as well as Akt phosphorylation and kinase activity. A PI3K inhibitor (LY294002) also reduced survivin promoter activity, implicating the PI3K/Akt pathway in survivin function. Upon treatment with 50  $\mu\text{M}$  EGCG, an increase in caspase-9 activation was seen in conjunction with a decrease in survivin expression over time (Tang *et al.*, 2007).

The effects of resveratrol, quercetin and catechin on cell proliferation and cell cycle of mammary tumors in nude mice xenografted with MBA-MD-231 cells were tested individually and in combination (Schlachterman *et al.*, 2008). Each of the 3 phytochemicals had no effect on cell proliferation individually at 0.5  $\mu\text{M}$ , but potently inhibited proliferation in combination, by 65% with each phytochemical at 0.5  $\mu\text{M}$ , by 83% with each at 5  $\mu\text{M}$ , and by 98% with each at 20  $\mu\text{M}$ . Individually, quercetin inhibited cell proliferation by ~60% and 90% at 5 and 20  $\mu\text{M}$ , respectively, while catechin inhibited proliferation by ~30% at 20  $\mu\text{M}$ . Individual phytochemicals had no effect on cell cycle at 0.5  $\mu\text{M}$ , but the combined treatments at 0.5  $\mu\text{M}$  each caused a statistically significant increase in S phase and concomitant decrease in G2/M phase cells. In doses of 20  $\mu\text{M}$ , resveratrol alone, quercetin alone, or the combination of the three caused a G0/G1 arrest, while catechin alone induced an S-phase accumulation.

In a study of the antiproliferative activities of tea catechins, (-)-catechin (400  $\mu\text{mol/mL}$ ) induced cell death in  $23.4\% \pm 1.7$  of the population of MCF-7 cells

(Friedman *et al.*, 2007). (-)-Epicatechin was much more potent, inducing cell death in  $22.3\% \pm 2.6$  of the population at 100  $\mu\text{mol/mL}$ ,  $64.7\% \pm 4.0$  at 200  $\mu\text{mol/mL}$ , and  $79.9\% \pm 19.4$  at 400  $\mu\text{mol/mL}$ . As mentioned earlier, both (-)-catechin and (-)-epicatechin were present in apple peels (He and Liu, 2008). After 48 hours of treatment, catechin (30 and 60  $\mu\text{g/mL}$ ) and procyanidin fractions (30  $\mu\text{g/mL}$  only) of grape seed extract reduced MCF-7 proliferation as measured by MTT assay and DNA synthesis (Faria *et al.*, 2006).

A study of catechins from litchi fruit extract compared cytotoxicities in MCF-7 breast carcinoma cells and in HELF human embryonic lung fibroblasts as the normal cell line (Zhao *et al.*, 2007). As a benchmark, cytotoxicity of paclitaxel was first measured. The  $\text{IC}_{50}$  of paclitaxel in MCF-7 cells was lower than 25  $\mu\text{g/mL}$ . At a dose of 100  $\mu\text{g/mL}$ , paclitaxel inhibited MCF-7 cells by 72.5% and HELF cells by 62.4%, not a very large margin. On the other hand, the  $\text{IC}_{50}$  of epicatechin was 102  $\mu\text{g/mL}$  in MCF-7 cells and 231  $\mu\text{g/mL}$  in HELF cells, and the  $\text{IC}_{50}$  of proanthocyanidin B2 was 99  $\mu\text{g/mL}$  in MCF-7 cells and 254  $\mu\text{g/mL}$  in HELF cells. The authors conclude that this large difference indicates these compounds may be effective at reducing proliferation of breast cancer cells while having low toxicity to normal tissues.

**c. Triterpenoids.** Among the 13 triterpenoids isolated from red delicious apples in our lab, four potently inhibited proliferation of MCF-7 cells with high selectivity (He and Liu, 2007). In other words, the  $\text{EC}_{50}$  for antiproliferative activity was low while the  $\text{CC}_{50}$  for cytotoxicity was high. A low selectivity index ( $\text{SI} = \text{CC}_{50}/\text{EC}_{50}$ ) indicates the effective antiproliferative doses begin approach cytotoxic doses. A safe, effective compound will have as high a selectivity index as possible. These triterpenoids were denoted as Compound 2 (2- $\alpha$ -hydroxyursolic acid), Compound 3 (3 $\beta$ -trans-cinnamoyloxy-2 $\alpha$ -hydroxyurs-12-en-28-oic acid), Compound

7 (2 $\alpha$ -hydroxy-3 $\beta$ -{[(2E)-3-phenyl-1-oxo-2-propenyl]oxy}olean-12-en-28-oic acid), and Compound 9 (3- $\beta$ -trans-p-coumaroyloxy-2- $\alpha$ -hydroxyolean-12-en-28-oic acid). For Compound 2, the EC<sub>50</sub> was 4.7  $\pm$  1.7  $\mu$ M, the CC<sub>50</sub> was 61.2  $\pm$  5.7  $\mu$ M, and the selectivity index (SI) was 13.0; Compound 3: EC<sub>50</sub> = 22.4  $\pm$  1.8  $\mu$ M, CC<sub>50</sub> >166.1  $\mu$ M, SI > 7.4; Compound 7: EC<sub>50</sub> = 29.2  $\pm$  3.3  $\mu$ M, CC<sub>50</sub> = 118.4  $\pm$  5.3, SI = 4.0; Compound 9: EC<sub>50</sub> = 20.9  $\pm$  2.3  $\mu$ M, CC<sub>50</sub> = 67.5  $\pm$  1.5, and SI = 3.2. By contrast, Compound 1 (ursolic acid) potently inhibited MCF-7 cells with an EC<sub>50</sub> of 14.4  $\pm$  1.8  $\mu$ M with a CC<sub>50</sub> of 18.2  $\pm$  0.6  $\mu$ M, giving an SI of only 1.3 (He and Liu, 2007).

2 $\alpha$ -Hydroxyursolic acid, one of the major triterpenoids isolated from apple peels, significantly inhibited MCF-7 cell proliferation (Yoon and Liu, 2008). In response to 2 $\alpha$ -hydroxyursolic acid treatment, the phosphorylation level of NF- $\kappa$ B inhibitor (I $\kappa$ B- $\alpha$ ) did not change, but proteasome activity in MCF-7 cells was inhibited significantly. These results suggest that 2 $\alpha$ -hydroxyursolic acid has antiproliferative activity against MCF-7 cells and the ability to inhibit NF- $\kappa$ B activation induced by TNF- $\alpha$  partially by suppressing proteasome activity.

**d. Dihydrochalcones.** As mentioned earlier, the main dihydrochalcones present in apples were phloretin 2'-glycoside (phlorizdin) and phloretin 2'-xyloglucoside (Tsao *et al.*, 2003). Phloretin and its glycoside, phloridzen, are known as effective inhibitors of glucose transport in the cell: phloretin is a general inhibitor of facilitated diffusion transporters (Krupka, 1985; Bakker *et al.*, 1999) and phloridzin is a competitive inhibitor of SGLT1 (Toggenburger *et al.*, 1982). Cancer cells can develop resistance to chemotherapeutics through overexpression of drug efflux proteins, such as P-glycoprotein and MRP1 (multidrug-resistance protein 1). Zhang *et al.* (2003) investigated the influence of various phytochemicals on this mechanism (biochanin A, morin, phloretin, and silymarin). For the purposes of this review, we

will limit the discussion to the results pertaining to phloretin, which is a phytochemical present in apples. In this study, 4 cell lines were used: MCF-7 (no detectable P-gp present), MDA435/LCC6WT (MDA/WT; wild type, moderate P-gp expression), MCF-7/ADR (overexpression of P-gp) and MDA435/LCC6MDR1 (MDA/MDR1; overexpression of P-gp). There was no significant expression of MRP1 in any of the cell lines. 50  $\mu$ M phloretin had no effect on [ $^3$ H]DNM (daunomycin) accumulation in MCF-7 cells as expected due to the lack of P-gp, but it significantly increase accumulation in MDA-WT and MCF7/ADR cells. In the MDA/MDR1 cells, phloretin non-significantly increased accumulation to 277% of control at 50  $\mu$ M and significantly increased accumulation to 431% of control at 100  $\mu$ M. The extent of increased DNM accumulation was associated with the extent of down-regulation of the P-gp. In MDA/MDR1 cells, phloretin actually decreased the cytotoxicity of doxorubicin, with an  $IC_{50}$  of 56.67  $\mu$ M, compared to the control, 30.18  $\mu$ M. In order to measure the effect of the phytochemicals on passive diffusion of the DNM into the cells, uptake rates were measured in the P-gp-negative MCF-7 cells. Phloretin did not increase passive diffusion but in fact slightly decreased the rate to 82.17% of control (50  $\mu$ M). 50  $\mu$ M phloretin did not significantly alter P-gp expression after a 2 hour incubation in MDA/MDR1 cells (110.2% of control), or a 24 hour pre-incubation in MCF7/ADR cells (83% of control). Phloretin did not significantly inhibit direct binding of substrate ([ $^3$ H]axidopine) with P-gp. Verapamil inhibits P-gp activity by increasing P-gp ATPase activity. Verapamil alone induced an ATPase activity of 32.6 and 31.7 in separate experiments; Phloretin alone induced activity to only 5.03. However, the combination of verapamil and phloretin induced ATPase activity to 52.74. The authors speculate the phloretin may bind to one of P-gp's 2 substrate binding sites or its allosteric site, altering the manner in which the verapamil interacts with the P-gp and thus enhancing its ATPase activity. The authors conclude



phytochemicals such as phloretin can interfere with P-gp-mediated drug efflux, which has implications for treatments, as well as drug interactions with dietary supplements of flavonoids (Zhang and Morris, 2003).

Another group also exploited phloretin's ability to inhibit glucose uptake to sensitize chemoresistant cells to daunorubicin (DNR) under hypoxic conditions (Cao *et al.*, 2006). Solid tumors often have hypoxic regions and are able to maintain high rates of glycolysis by overexpressing glucose transporters and glycolytic enzymes. Hypoxia and high expression of glucose transporters are associated with chemoresistance in cancer cells. The researchers measured cytotoxicity of DNR with and without phloretin, under normoxic and hypoxic conditions. In SW620 colon cancer cells, the  $IC_{50}$  under normoxia was 11 nM and under hypoxia was 50 nM. Addition of 50  $\mu$ M phloretin to the DNR resulted in an  $IC_{50}$  of 14 nM under normoxia and 25 nM under hypoxia, thus sensitizing the cells to the effects of DNR under hypoxic conditions only. A similar trend was seen in K562 drug-sensitive leukemia cells. The  $IC_{50}$  of DNR under normoxia was 21 nM and under hypoxia was 44 nM. Addition of phloretin changed the  $IC_{50}$  to 35 nM under normoxia and 20 nM under hypoxia. Similar effects were seen in K562/Dox cells, a drug-resistant leukemia cell line that overexpresses P-gp: Under normoxia, phloretin alone did not kill cells and addition of phloretin to DNR did not enhance the drug's cytotoxicity. However, under hypoxia, addition of 50  $\mu$ M phloretin to 1  $\mu$ M DNR increased cytotoxicity from 40-50% to 70%. Apoptosis was also increased under the hypoxic conditions (phloretin induced no significant apoptosis; 1  $\mu$ M DNR induced 32% apoptosis; combination induced 63% apoptosis; Annexin-V staining). The authors note such differences in effects under normoxic and hypoxic conditions must be kept in mind when designing *in vitro* assays measuring efficacy of chemotherapeutic agents. Also, they point out

that the specificity of the effects of phloretin in combination with DNR on hypoxic cells versus normoxic cells may confer tumor selectivity *in vivo*, a desirable characteristic of any chemotherapeutic treatment (Cao *et al.*, 2006).

In a review of the anti-cancer activity of chalcones (Go *et al.*, 2005), phloretin was cited as having antioxidant activity in a DPPH free radical scavenging assay measured by electron spin resonance. The antioxidant activity was attributed to the hydroxyl groups (Anto *et al.*, 1995). In doses up to 50  $\mu$ M, phloretin was found to have no effect on proliferation of MCF-7 cells (Calliste *et al.*, 2001).

Phloridzen, a glycoside of phloretin, was shown to increase absorption of genistin by 2.5-fold in a rat small intestine perfusion model (Andlauer *et al.*, 2004). Phloretin and phloridzin both inhibited proliferation of Fisher bladder cell carcinoma *in vitro*, as well as in a rat mammary adenocarcinoma model after intraperitoneal administration (Nelson and Falk, 1993).

## V. IGF SIGNALING

**A. The IGF System.** IGF-I (Insulin-like Growth Factor-I) is a peptide hormone which acts as a mediator between growth hormone (GH) and growth during fetal and early childhood development (Holly *et al.*, 1999). Insulin-like Growth Factor I (IGF-I) is produced by the liver for endocrine functions (Yakar *et al.*, 1999) and by many other tissues for autocrine and paracrine functions (Adamo *et al.*, 2005). The three major factors influencing endocrine IGF-I production in the liver are growth hormone (Bichell *et al.*, 1992), insulin (Kaytor *et al.*, 2001; Kaytor *et al.*, 2001b) and nutritional status (caloric and protein intakes, Clemmons *et al.*, 1981; Thissen *et al.*, 1994). IGF-I acts through binding to the IGF-I Receptor (IGF-IR). The IGF-IR is

70% homologous to the insulin receptor, but has independent roles in cell proliferation and survival, differentiation, and neoplastic growth (Baserga *et al.*, 2003).

**1. Components of the IGF System.** The IGF system is comprised of 2 growth factors (IGF-I and IGF-II), 3 receptors (the IGF-I Receptor, IGF-IR; the mannose-6-phosphate/IGF-II Receptor, IGF-IIR; and the Insulin Receptor, IR), and 6 IGF binding proteins (IGFBP-1 through -6) (Adamo *et al.*, 2005). IGF-I signals primarily through the IGF-IR, a tyrosine kinase receptor made of 2 extracellular  $\alpha$  subunits and 2 transmembrane  $\beta$  subunits. The  $\beta$  subunits are 84% homologous to the IR at the amino acid level. IGF-I may bind to the insulin receptor, but with 100 to 1,000-fold lower affinity than insulin, and with half the affinity it has for the IGF-IR. IGF-I has much lower affinity for the IGF-IIR than does IGF-II. IGF-II can elicit mitogenic effects by binding to the IGF-IR or to the IR. Specifically, IGF-II has a high affinity for the IR-A splicing variant but none at all for the IR-B variant (Adamo *et al.*, 2005). The role of the IGF-IIR in this system is to internalize and degrade IGF-II, thus removing this growth factor from circulation. Insulin does not bind to the IGF-IR; however, it may bind to an IGF-IR/IR hybrid, but with a lower affinity than IGF-I has for the hybrid (Ryan and Goss, 2008). IGFBPs bind the IGFs in the serum, protecting them from degradation and extending the half-life. Locally, BPs may either inhibit or enhance IGF action. Most of the circulating IGF-I is bound to IGFBP-3 and acid-labile subunit (ALS) in a ternary complex (Adamo *et al.*, 2005 and sources therein).

**2. Normal physiological roles of the IGF system and the influence of nutrition.** Somatic growth, from fetus to adult, is regulated mainly by the IGF system. As the IGF-I system is highly influenced by nutrition, somatic growth of the fetus is regulated by both fetal IGF-I levels and maternal nutrition (Blanco and Ferry, 2005). Compared to premature newborns, term newborns have higher IGF-I and -II levels, which are good indicators of weight and length at birth as well as gestational age

(Prosser, 1996). IGF-II levels are higher than IGF-I in the fetus, while IGF-I levels are higher than IGF-II after birth. In the fetus, IGFBP-1 and -2 are the main IGF binding proteins present in serum, and decrease over the gestational period as IGFBP-3 and ALS increase to become the dominant binding proteins in postnatal life (Prosser, 1996; Blanco and Ferry, 2005).

Human breast milk contains IGF-I and -II, and IGFBPs (Smith *et al.*, 1997; Blanco and Ferry, 2005), and is believed to promote the maturation of the newborn's incompletely developed intestinal epithelium (Blanco and Ferry, 2005). At birth, there is a positive association between the IGFI:IGFBP-3 ratio and weight, length, and head circumference (Prosser, 1996; Blanco and Ferry, 2005). IGF production only becomes dependent on GH stimulation after 6 months of age (Prosser, 1996; Blanco and Ferry 2005). In the newborn, feeding stimulates release of insulin which lowers IGFBP-1 release, thereby increasing circulating levels of available IGF-I to promote growth (Bhala *et al.*, 1998; Blanco and Ferry, 2005). Similarly, in adults and children, fasting or decreasing calories or protein will lower total serum IGF-I levels (Clemmons *et al.*, 1981; Isley *et al.*, 1983; Thissen *et al.*, 2005). Protein and caloric intake influence IGF-I levels in premature infants (Donovan *et al.*, 1991; Blanco and Ferry, 2005).

The IGF system is vital to the reproductive system. Yet, it does not work in isolation but rather as part of a network including GH, steroid hormones (estrogen, progesterone, and testosterone) and nutrition to support normal reproductive function and successful pregnancies. The ovaries have a self-contained, paracrine IGF system necessary for proper ovulation, circulating reproductive hormone concentrations and early embryo development (Zhou *et al.*, 1997; Simmen *et al.*, 2005). Circulating endocrine IGF and nutrition also impact ovary function, the plausible connection being that an undernourished female cannot support a pregnancy. Therefore, ovary

function may be turned off, in response to under-nutrition and decreased IGF-I, at various stages of the process, such as hormone secretion, ovulation, corpus luteum development, and survival of the embryo (Simmen *et al.*, 2005).

The fallopian tubes and uterus also have local IGF systems critical to their normal functions. IGF-I and nutrition may affect these organs although currently the evidence is unclear (Simmen *et al.*, 2005). IGF function in the male reproductive system has been less extensively studied. Some indications of a role for IGF and nutrition in the testis come from animal studies. Increased feeding in rams is associated with increased testis size, plasma testosterone and IGF-I, peak pubertal reproductive activity and increased body weight (Adam and Findlay, 1997; Simmen *et al.*, 2005). Bovine sperm cells express IGF-IR, and IGF-I and -II increase bovine sperm motility (Henricks *et al.*, 1998; Lackey *et al.*, 1998; Simmen *et al.*, 2005). In summary, while the reproductive system is influenced by endocrine IGF-I and nutrition, it is mostly under the control of local paracrine IGF systems.

Over the lifespan, IGF-I levels fall with age, and IGF-BPs fall to a lesser extent, allowing a higher proportion of circulating IGF-I to be bound to the BPs and therefore less bioavailable (Lewitt and Hall, 2005). It is not clear whether the main contributing factor is reduced GH levels with age, changes in nutrition over time, or both (Lewitt and Hall, 2005). Higher than normal range IGF-I is seen in individuals living longer than 100 years (Paolisso *et al.*, 1997; Lewitt and Hall, 2005). IGF-I may play a role in many diseases of aging. For example, lower levels of IGF-I are associated with dementia (Arai *et al.*, 2001; Lewitt and Hall, 2005) and reduced immune function (Krishnaraj *et al.*, 1998; Lewitt and Hall, 2005). The IGF system may play important roles in osteoporosis, muscle wasting, and cardiovascular disease in aging (Lewitt and Hall, 2005).

IGF-I contributes to bone formation and maintenance of bone density throughout life by activating osteoblast cells (Thissen *et al.*, 1994; Bonjour *et al.*, 2005) increasing reabsorption of inorganic phosphate (Pi) by the kidney (Bonjour *et al.*, 1991; Bonjour *et al.*, 2005), and producing calcitriol, the hormonal form of Vitamin D, (Caverzasio and Bonjour, 1991; Bonjour *et al.*, 2005) which is responsible for intestinal absorption of calcium and Pi. These normal functions of IGF-I in relation to bone health are blocked in the face of protein deficiency. (Decreased dietary protein lowers GH secretion, and consequently IGF-I secretion is reduced.) For example, a protein-deficient diet is common among elderly hip fracture patients (Older *et al.*, 1980; Bonjour *et al.*, 2005). Upon protein supplementation, circulating IGF-I levels increased as did bone mass and muscle mass. Consequently, risks of falling, further fractures, medical complications, and deaths were reduced (Delmi *et al.*, 1990; Bonjour *et al.*, 2005). Decreased protein intake influences bone loss through two GH-dependent routes: (1.) by decreasing IGF-I production and action, thereby reducing bone formation, and (2.) by decreasing sex hormone production and increasing osteolytic cytokines thereby increasing bone resorption (Bonjour *et al.*, 2005).

IGF-I aids in skeletal and cardiac muscle protein synthesis and maintenance. Catabolic conditions such as critical illness, AIDS, alcohol intoxication, burns, sepsis and endotoxemia see lowered levels of IGF-I expression and activity. Some of these conditions show a favorable response to treatment with IGF-I (Lang and Frost, 2005). IGFs play important roles in the generation and protection of neurons, both in normal function and in a clinical setting as a treatment for head injury and nerve damage. Growth and interaction between neurons and muscle cells is maintained in part by IGF-I. The effects of nutrition on IGF-I's role in the nervous system need to be further studied (Meyer *et al.*, 2005). The kidney expresses a local IGF system. IGF-I plays a

role in renal growth, renal blood flow, glomerular filtration rate, and renal transport of phosphate and sodium, as well as in compensatory renal growth and renal hypertrophy in several diseases of the kidney (Rabkin *et al.*, 2005).

The IGF system is essential to normal mammary gland development and function. Briefly, pituitary GH stimulates mammary development during puberty. GH induces IGF-I production in the stroma of the mammary gland, and subsequently IGF-I and E2 work together to promote proliferation of terminal end buds (TEBs) which extend, filling the mammary fat pad with a network of branching ducts. During pregnancy, IGF-I is necessary for progesterone to stimulate lobulo-alveolar development (Kleinberg and Ruan, 2008 ).

Rowzee *et al.* (2008) review this topic in more detail. Evidence suggests that in rat mammary glands, ductal branching relies on locally-produced IGF-I rather than circulating IGF-I. In humans, IGF-II may also be important in mammary tissue development. In response to GH, IGF-I is expressed in the mammary stroma throughout post-natal development and regulates expression of S and G2 cyclins. IGF-I and IGF-II expression occurs in TEBs during puberty and promotes ductal branching. In the post-pubertal and early pregnancy epithelium, IGF-II is expressed while IGF-I is not. IGF-I expression returns to the ductal epithelium during mid-through late-pregnancy, and to the alveolar epithelium during late pregnancy. IGF-II expression (regulated mostly by prolactin) maintains a non-uniform expression pattern in the ductal epithelium in post-puberty as well as in the alveoli during pregnancy. There are difficulties in discerning distinct roles of IGF-I and II. It is clear local IGF-II cannot rescue pubertal mammary epithelial growth in the absence of IGF-I. However, IGF-II does have an essential role in prolactin-mediated alveolar development.

IGFBPs, expressed in both the epithelium and stroma, work to regulate and partition the IGFs in the mammary tissue (Rowzee *et al.*, 2008).

The IGF-IR is expressed in the mammary epithelium during all developmental stages and at lower levels in the stroma during periods of growth (puberty, post-puberty, and pregnancy). It appears the IGF-I expressed in the stroma works in a paracrine fashion on the epithelial receptors to induce proliferation. It is possible the stromal receptors may play a role as well. Over-expression or over-activation of IGF-IR may lead to hyperplasias and tumor formation in mammary epithelium. IR isoforms have been detected in mammary epithelial cells and breast cancer cell lines, and are critical in the development of mammary epithelial cells, particularly the differentiation of alveoli. Expression patterns of IR during various stages of development are not as defined as for the IGF-IR. IGF-II may mediate functions in mammary tissues through both the IGF-IR and the IR-A. Hybrid receptors containing one IGF-IR  $\alpha$ - $\beta$  subunit and one IR  $\alpha$ - $\beta$  exist in mammary tissues. These hybrid receptors have higher affinity for IGF-I than for insulin, however the exact functions and downstream signaling pathways are unknown. In humans, the expression of IR isoforms in normal breast epithelium, as well as whether IGF ligand expression patterns mirror those seen in rodents, are unknown (Rowzee *et al.*, 2008).

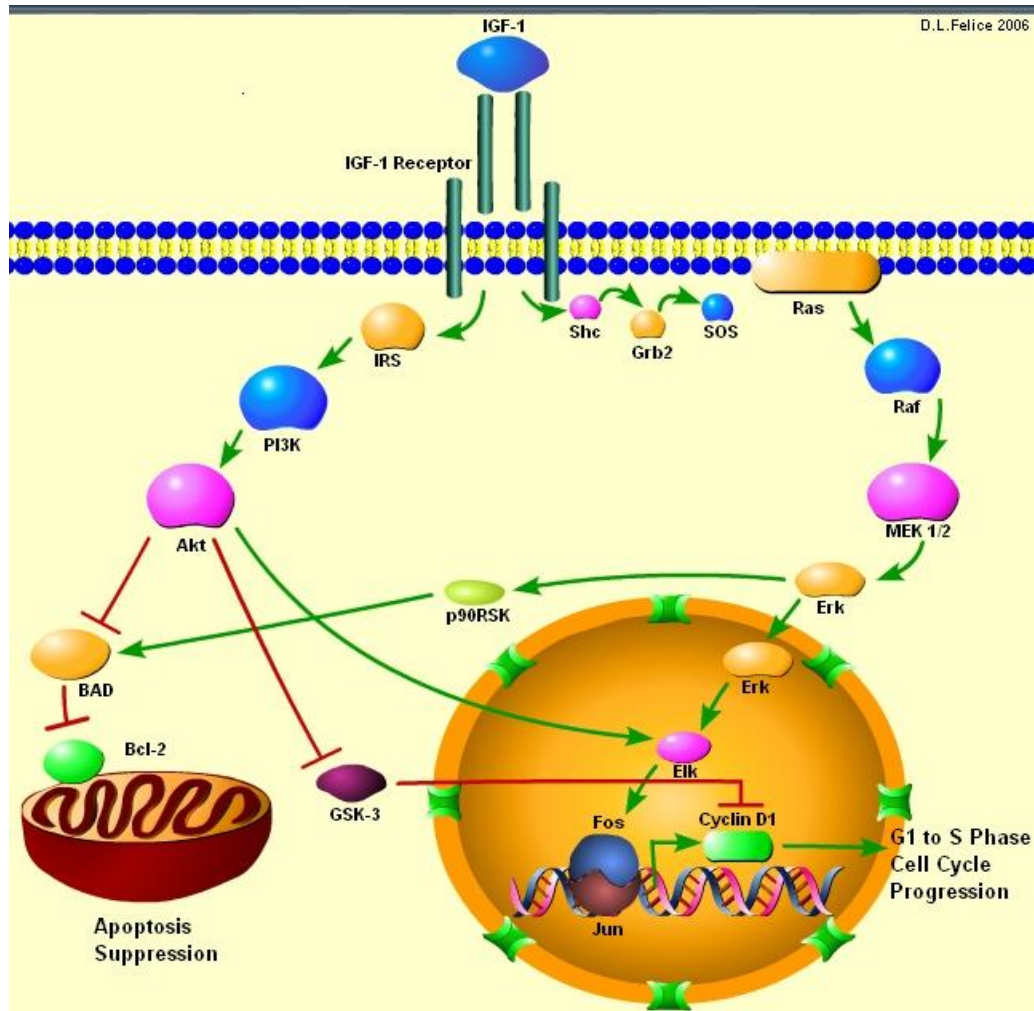
**3. PI3K Pathway (Figure 1.1).** The IGF-IR signals through two major pathways: the PI3K/Akt pathway, and the Mek/Erk MAPK pathway (Werner and LeRoith, 2000). In MCF-7 cells, the IGF-IR exerts its mitogenic effects through the PI3K/Akt pathway (Dufourny *et al.*, 1997), which was the focus of this study. Binding of IGF-I to the receptor stimulates autophosphorylation of the receptor which then phosphorylates Insulin Receptor Substrate-1 (IRS-1) (Izumi *et al.*, 1987; Shemer *et al.*, 1987). Phosphorylation of IRS-1 creates a docking site suitable for Src-homology-domain-containing signaling proteins such as the p85 subunit of PI3K. Through



phosphorylation of precursor molecules in the lipid membrane, PI3K is responsible for the generation of phosphatidylinositol-3, 4, 5-triphosphate (PIP3), which recruits other molecules such as PDK-1 and Akt. PDK-1 is then able to phosphorylate and activate Akt, which is responsible for activating a myriad of downstream signaling pathways (Lawlor and Alessi, 2001; Cantley, 2002). As a result of IGF-IR signaling, Akt drives cell cycle progression by promoting transcription of Cyclin D1 as well as preventing Cyclin D1 degradation by inhibiting GSK-3 (Lawlor and Alessi, 2001). Akt suppresses apoptosis by phosphorylating and thus inhibiting the pro-apoptotic mitochondrial protein Bad (Downward, 1999), and phosphorylating and inactivating Caspase-9 (Cardone *et al.*, 1998). In order to be fully activated, Akt must be phosphorylated on two key residues: Ser<sup>473</sup> and Thr<sup>308</sup>. Thr<sup>308</sup>, which occurs in the activation loop of the protein, is phosphorylated by PDK-1 (Alessi *et al.*, 1996). mTOR phosphorylates Ser<sup>473</sup>, in the carboxy terminus (Sarbasov *et al.*, 2005). Phosphorylation of both of these residues is required for full Akt activation, both are stimulated by IGF-I treatment, and phosphorylation of one is not dependent on the phosphorylation state of the other (Alessi *et al.*, 1996).

**4. MAPK Pathway (Figure 1.1).** Shc proteins (cytoplasmic tyrosine kinases) bind to the Tyr<sup>950</sup> residue of the IGF-IR and are activated. Shc proteins then bind Grb2, an adaptor protein that also binds SOS. In this way, SOS, a guanine nucleotide exchange factor (GEF), activates Ras. Activated Ras sets off the MAPK cascade that is comprised of Ras (monomeric GTP-ase), raf (Mitogen activated protein kinase kinase kinase, MAPKKK), MEK (MAPK/ERK kinase, MAPKK), and ERK (Extracellular signal-regulated kinase, MAPK). Activated ERK translocates to the nucleus where it activates the transcription factor Elk-1, which drives transcription of the AP-1 transcription factor, responsible for Cyclin D1 transcription. Peruzzi *et al.* (1999) found that sustained MAPK activation (at least 1 hour) was required to sustain

Bad phosphorylation. In addition, inhibition of the MAPK pathway resulted in a decrease in the level of Bad phosphorylation.



**Figure 1.1. IGF-I Receptor-Mediated Signaling Pathways.**

**5. Estrogen Receptor Signaling.** ER $\alpha$  functions via genomic events in the nucleus, and via signal transduction events in the plasma membrane (Mawson *et al.*, 2005). The ER and IGF-IR pathways collaborate to promote cell cycle progression in various ways. In the nucleus, estrogen has been shown to increase the transcription of IGF-I, IGF-IR, and IRS-1. IGF-I-treated ER $^{+}$  MCF-7 cells demonstrated increased ER transcriptional activity. Estrogen increases expression of c-myc, which blocks p21,

while insulin increases expression of Cyclin E. Estrogen, insulin, and IGF-I also promote expression of Cyclin D1. Together these events lead to enhanced progression through the G1-S transition. At the plasma membrane, estrogen-bound ER may bind and activate the IGF-IR or bind the p85 subunit of PI3K and Src, directly activating the PI3K and p21/ras/MAPK pathways (Mawson *et al.*, 2005).

Since the ER $\alpha$  lacks a transmembrane domain, Song *et al.* (2004) investigated whether the IGF-IR may play a role in the translocation of ER $\alpha$  to the plasma membrane based on the following evidence: ER $\alpha$  and IGF-IR are co-expressed, there is crosstalk among their proliferative and anti-apoptotic signaling pathways, and E2 stimulated IGF-IR activation and binding of ER $\alpha$  to Shc. Using immunoprecipitation assays, the authors found that in cells treated with 0.1 nM E2, ER $\alpha$  and Shc associated with the IGF-IR in a time-dependent manner. In the absence of E2, Shc associated with IGF-IR but ER $\alpha$  did not. IGF-I (1 ng/mL) did not induce association of ER $\alpha$  with the IGF-IR. The ternary complex of ER $\alpha$ , Shc and IGF-IR was disrupted by addition of the antiestrogen compound ICI 182,780. The use of inhibitors (AG1024 for IGF-IR, ICI 182,780 for ER $\alpha$ , PP2 for Src) demonstrated that ER $\alpha$  and c-Src regulated E2's ability to activate IGF-IR. Demonstrated by immunoprecipitation, knockdown of Shc using a specific siRNA caused an 87% decrease in the association between ER $\alpha$  and IGF-IR (both basal levels and E2-induced). Then, using confocal analysis, the authors showed that Shc was located in the cytoplasm and ER $\alpha$  in the nucleus. Addition of E2 caused the translocation of both of these proteins to the plasma membrane, which was blocked by knockdown of Shc with siRNA. Total expression of ER $\alpha$  did not change, and neither did the effects of ER $\alpha$ -mediated changes in cell morphology induced by E2, such as ruffles and filopodia. Knockdown of IGF-IR with siRNA inhibited the ability of ER $\alpha$  to translocate to the cell membrane, but had no effects on morphologic changes induced by E2. Inhibition of each of the three proteins (IGF-IR, ER $\alpha$ , and

Sch) by either siRNA or chemical inhibitors blocked E2-induced MAPK phosphorylation. Additionally, treatment with the IGF-IR inhibitor blocked E2-induced cell proliferation. The authors note that ER $\alpha$  has been shown to associate also with the p85 domain of PI3K which may also play a role in the translocation of ER $\alpha$  to the plasma membrane. Focusing on the results of the current study, Song *et al.* propose the following model: E2-stimulated interaction between ER $\alpha$  and Shc activates Src. Src phosphorylates and activates IGF-IR which recruits the Shc. Shc interaction with IGF-IR brings the ER $\alpha$  to the plasma membrane and activates the MAPK pathway (Song *et al.*, 2004). This model may partly explain the mechanism by which breast cancer cells develop resistance to tamoxifen (Fagan and Yee, 2008).

Fagan and Yee (2008) review recent findings illustrating the interactions between ER and IGF-IR signaling. ER can be activated by phosphorylation induced by estrogen or other growth factors such as EGF and IGF-I (Kato *et al.*, 1995; Bunone *et al.*, 1996; Fagan and Yee, 2008). Activation function domain-1 (AF-1) of the ER is regulated by growth factors, while AF-2 is activated by estrogen. IGF-I can induce ER activation in two ways: phosphorylation of Ser<sup>118</sup> of the AF-1 by MAPK, and phosphorylation of Ser<sup>167</sup> of AF-1 by Akt (Ignar-Trowbridge *et al.*, 1993; El-Tanani and Green, 1997).

In both normal and cancerous breast tissue, IGF-I and estrogen both work to potentiate one another's transcriptional activity (Fagan and Yee, 2008). In the absence of estrogen, treatment with IGF-I increased transcriptional activity of the ER in MCF-7L cells (Lee *et al.*, 1997). IGFBP-1 inhibited ER activation by both IGF-I and by estrogen (Figueroa *et al.*, 1993a). The implication is that IGF-I is necessary for the ER to reach its full activation potential stimulated by estrogen (Fagan and Yee, 2008).

IGF-I can contribute to ER signaling, while estrogen can contribute to IGF-IR signaling. Evidence to support this claim includes the following: IGF-I-stimulated growth is inhibited by anti-estrogens, and estrogen-stimulated growth is inhibited by anti-IGF-I treatments (Wakeling *et al.*, 1989; Freiss *et al.*, 1990). In breast cancer cells and/or MCF-7L xenografts, estrogen can increase expression and phosphorylation of IGF-IR and IRS-1, increase phosphorylation of MAPK, increase IGF-II expression, and decrease expression of IGFBP3 and IGF-IIR (Osborne *et al.*, 1989; Mathieu *et al.*, 1991; Lee *et al.*, 1994; Huynh *et al.*, 1996; Nickerson *et al.*, 1997; Lee *et al.*, 1999). Anti-estrogens increase IGFBP3 expression (Figuerola *et al.* 1993b; Huynh *et al.*, 1996), and decrease expression and activation of IGF-IR and IRS-1 (Stewart *et al.*, 1992; Salerno *et al.*, 1999). Additionally, estrogen and IGF-I have several transcriptional targets in common, such as c-myc, c-fos, c-jun, cyclin D1 and p21 (Dubik and Shiu, 1992; Musgrove and Sutherland, 1994; Morishita *et al.*, 1995).

Cascio *et al.* (2007) investigated the effects of IGF-I and estrogen on transcriptional activity of ER. In MCF-7 cells, treatment for 1 or 4 hr with IGF-I alone (50 nM) resulted in significantly less nuclear translocation of the Estrogen Receptor (ER) than treatment with E2 alone (10 nM) (Cascio *et al.*, 2007). Combined treatment resulted in slightly less nuclear translocation after 1 hr and slightly more after 4 hr compared to the E2 alone. Treatment with IGF-I alone stimulated recruitment of p300 and SRC-1 to the Cyclin D1 AP-1 sequence and expression of Cyclin D1 mRNA (expression was highest when treated with IGF-I, compared to E2 or both together). IGF-I also stimulated recruitment of ER and co-regulators of ER to the AP-1 site (Carm, Mdm2 and E6L). In the face of ER knockdown by RNA interference, IGF-I was still able to recruit p300 and SRC-1 and partially express Cyclin D1, but loading of ER, E6L, Mdm2, Carm and polII on the AP-1 sequence was reduced. This paper provides further evidence of the crosstalk between IGF-I and ER signaling. While

IGF-I can stimulate nuclear localization of the ER, it is less efficient than E2. After 1 and 4 hrs of treatment, IGF-I stimulated transcription of Cyclin D1 mRNA more efficiently than E2 or both in combination. IGF-I recruited ER to the AP-1 sequence to fully activate transcription of Cyclin D1, although IGF-I alone recruited less ER than E2 had recruited. However, even in the absence of the ER, IGF-I was able to stimulate some Cyclin D1 transcription (Cascio *et al.*, 2007).

**B. IGF-I and Breast Cancer.** Nutrition, the IGF-I system, and cancer risk are all closely intertwined (Houston *et al.*, 2005). Caloric restriction studies in a p53-deficient mouse bladder cancer model, which reduced cancer incidence, were also shown to reduce IGF-I levels (Dunn *et al.*, 1997). Adding IGF-I back to the circulation reversed the effect on cancer incidence. Higher levels of GH are associated with higher cancer risk (Maison *et al.*, 1998). Circulating levels of IGF-I, controlling for the IGFBP3 binding protein, have been shown to correlate more significantly with hormone responsive breast cancer than circulating levels of estrogen, controlling for SHBG (sex hormone binding globulin) (Holly *et al.*, 1999). In premenopausal women, IGF-I/IGFBP3 levels correlate with high mammographic breast density (Byrne *et al.*, 2000), which in turn is known to correlate with increased breast cancer risk (Hankinson *et al.*, 1998). The IGF-IR has been shown to be overexpressed in both breast cancer tumors and cell lines (Lann and LeRoith, 2008).

Lann and LeRoith (2008) review various *in vivo* studies that illustrate the interdependence among growth hormone (GH), IGF-I, and breast cancer development, progression, and aggressiveness. For example, a transgenic mouse that overexpresses a form of IGF-I with reduced affinity for IGFBP-3 had higher occurrence of mammary intraepithelial neoplasia and tumor development (Hadsell *et al.*, 2000). Another mouse model, in which the GHRH (Growth Hormone Releasing Hormone) gene was knocked out resulting in circulating levels of GH and IGF-I that were about 10% of

normal, had significantly smaller mammary tumors compared to control mice after injection of MCF-7 cells into the mammary fat pads (Yang *et al.*, 1996). Spontaneous Dwarf Rats, deficient in GH and IGF-I as a result of a *gh* gene mutation, are immune to chemically-induced mammary cancer (Swanson and Unterman, 2002). Replacing GH or IGF-I increased the incidence of mammary tumor development in these rats (Thordarson *et al.*, 2004). The Liver IGF-I-Deficient (LID) mouse model, which reduces circulating endocrine IGF-I to 25% of normal, demonstrates reduced mammary cancer incidence (26% of animals) and delayed latency period (74 days) in response to DMBA treatment compared to control animals (56% developed tumors, latency period of 59.5 days, Wu *et al.*, 2003).

**C. Phytochemicals and IGF-IR Signaling.** Synergistic effects of soy and tea phytochemicals were tested in a mouse model in which MCF-7 cells were implanted in the mammary fat pads of SCID (severe combined immune deficient) mice with estrogen supplementation to support MCF-7 cell growth (Zhou *et al.*, 2004). Dietary interventions were initiated 2 weeks prior to implantation and continued throughout the experiment (8 weeks post-implantation). Dietary interventions were soy phytochemical concentrate (SPC, 51.9% soy isoflavones by weight), genistein-rich soy isoflavone mixture (GSI, 100% soy isoflavones by weight), and green tea or black tea infusions (1.5%, 1.5g/100 mL water). GSI and SPC reduced tumor size and volume in a dose-dependent manner. Green tea (GT) also decreased tumor weight and volume, but black tea (BT) or 0.1% SPC did not. Interestingly, a combination of 0.1% SPC with either GT or BT reduced tumor weight and volume to a greater extent, indicating some synergistic activity. BT treatment reduced ER $\alpha$  expression in the MCF-7 cells, as did the 0.1% SPC/GT combination. However, ER $\alpha$  expression was unaffected by other individual treatments (SPC, GSI, GT, or 0.1% SPC). At the end of

the 8 week experiment, the researchers measured plasma levels of IGF-I. 0.1% SPC, BT, and SPC/BT actually increased circulating IGF-I by 11.1, 5.5 and 4.1%, respectively. GT treatment decreased IGF-I by 19.0% ( $p > 0.05$ ), while the SPC/GT combination significantly reduced IGF-I even further, by 35.8% ( $p < 0.05$ ). The authors conclude that in this mouse mammary cancer model, phytochemicals from soy and green tea work synergistically to inhibit MCF-7 tumor cell growth by down-regulating ER $\alpha$  expression and reducing circulating levels of IGF-I (Zhou *et al.*, 2004).

Phytochemical soy concentrate (but not treatment with pure genistein) reduced lung metastases by 95% in a mouse lung cancer model by reducing circulating IGF-I levels and down-regulating NF $\kappa$ B expression (Singh *et al.*, 2006).

Phytochemicals have been shown to modulate cell cycle and induce apoptosis via IGF-IR-mediated pathways. For example, in the rat prostate cancer cell line AT6.3, phytochemicals commonly found in tomato and soy interfered with IGF-IR-stimulated cell survival (Wang *et al.*, 2003). Genistein, biochanin A, quercetin, kaempferol, daidzein and rutin all inhibited IGF-I-stimulated cell proliferation with varying potencies. While IGF-I increased the proportion of cells in S-phase, genistein and kaempferol caused a G2/M arrest. Quercetin and biochanin A caused a less dramatic G2/M arrest. Daidzein and rutin did not appear to affect cell cycle distribution. Genistein, biochanin A, quercetin and kaempferol all induced apoptosis in IGF-I-treated cells, as was demonstrated by the sub-G0 peak in cell cycle analysis, Annexin V staining, DAPI staining, and the TUNEL assay. Compared to IGF-I control (50  $\mu$ g/L), 50  $\mu$ mol/L quercetin in addition to the IGF-I significantly reduced Akt phosphorylation, and insignificantly increased ERK 1/2 phosphorylation. In this study, both Akt and ERK 1/2 activation were shown to play a role in proliferation of the AT6.3 cell line (Wang *et al.*, 2003).



Doses of 25 through 100  $\mu$ M quercetin inhibited proliferation of PC-3 cells, an androgen-independent prostate carcinoma cell line, after 24, 48 and 72 hours of exposure (Vijayababu *et al.*, 2006). After 24 and 48 hours, quercetin increased IGFBP3 secretion, and decreased secretion of IGF-I and IGF-II. Quercetin up-regulated Bax and caspase-3 expression and down-regulated Bcl-2 and Bcl-x<sub>L</sub> after 24 hours of treatment. According to sub-G0 fragment analysis, DNA fragmentation assay and the TUNEL assay, 50 and 100  $\mu$ M quercetin induced apoptosis of PC-3 cells.

IGF-I can rescue MCF-7 cells from death in the face of protein synthesis inhibition (Geier *et al.*, 1995). Treatment of MCF-7 cells with cycloheximide (CHX), a protein synthesis inhibitor, caused cell death which began with apoptosis and ended with necrosis. In the presence of 30  $\mu$ g/mL CHX and continued protein synthesis inhibition, 10 ng/mL IGF-I rescued the cells. Genistein, a tyrosine kinase inhibitor, demonstrated a dose-response (12-96  $\mu$ g/mL) in blocking the survival effect of IGF-I. The authors concluded the IGF-I triggered some survival mechanism that does not rely on protein synthesis, but rather through some post-translational modifications such as phosphorylation (Geier *et al.*, 1995).

Nordihydroguaiaretic acid (NDGA), a phenolic compound found in the creosote bush *Larrea divaricata*, inhibits activation of the IGF-IR, and subsequent phosphorylation of downstream proteins Akt and BAD in MCF-7 cells (Youngren *et al.*, 2005). Pretreatment of isolated IGF-IR preparations with NDGA for 20 minutes prior to treatment with 10 nM IGF-I reduced tyrosine kinase activity of the IGF-IR in a dose-dependent manner (0.3 to 100  $\mu$ M NDGA). Similarly, pre-treatment of MCF-7 cells for 1 h with NDGA before addition of 3 nM IGF-I reduced autophosphorylation of IGF-IR in a dose-dependent manner (7.5 to 45  $\mu$ M NDGA). One hour pre-incubation with varying doses of NDGA (7.5 to 90  $\mu$ M) prior to 10 minutes treatment with 3 nM IGF-I reduced Akt phosphorylation and BAD phosphorylation in a dose-

dependent manner. After 3 days of incubation, NDGA significantly reduced MCF-7 cell proliferation in basal medium containing 10% FCS in a dose-dependent manner. In medium containing 10 nM IGF-I and no other growth factors, NDGA also reduced proliferation in a dose-dependent manner, more potently than FCS-stimulated cells. The researchers also tested the effects of NDGA *in vivo*. MCNueA breast cancer cells were injected into female neuTg mice, and were given NDGA either orally or intraperitoneally from day 9 after implantation of cells until the end of the experiment at day 29. While the NDGA treatment did not alter the cellular expression of the IGF-IR nor the HER2/neu receptor, treatment reduced autophosphorylation of both receptors and reduced tumor growth rate. Interestingly, the results were the same whether the NDGA was administered orally or intraperitoneally (Youngren *et al.*, 2005).

When added to IGF-I-treated MCF-7 cells, curcumin reduced cell numbers in a time- and dose-dependent manner (24 and 48 h; 10, 20 and 40  $\mu$ M Curcumin). After 24 hours in regular medium, curcumin caused a G2/M arrest with a concomitant increase in sub-G0 peak and decrease in S phase cells, indicating cell cycle blockage followed by apoptosis. Flow cytometry, Annexin V staining, and observation of nuclear morphology showed that IGF-I suppressed apoptosis in the MCF-7 cells, while the curcumin was able to reverse this protective effect. Curcumin also reduced secretion of IGF-I, and increased secretion of IGFBP3. In addition, the curcumin reduced IGF-IR tyrosine kinase activity as well as IGF-IR mRNA expression (Xia *et al.*, 2007).

Kaempferol inhibited proliferation of ER(+) MCF-7 cells (IC<sub>50</sub> of 35  $\mu$ M at 48 hours) via a down-regulation of ER- $\alpha$ , progesterone receptor, Cyclin D1, and IRS-1, and reduced activity of the estrogen-responsive-element-reporter gene. Kaempferol

also inhibited proliferation of ER(-) cells but less potently (IC<sub>50</sub> of 70  $\mu$ M at 48 hours) (Hung, 2003).

MCF-7 cells were treated for 1 to 3 days with doses of lycopene ranging from 0.75 to 3  $\mu$ M in medium containing either a standard concentration of FCS (3%), or 0.5% FCS with 30 nM IGF-I. Both the [3H]thymidine incorporation assay and direct cell counting using a Coulter counter showed that lycopene inhibited cell proliferation in both types of medium, but inhibited the IGF-I-stimulated cells to a greater extent (Karas *et al.*, 2000). The study went on to analyze cell cycle distribution, cell death, and IGF-IR signal transduction. Flow cytometric analysis revealed lycopene treatment slowed cell cycle progression in IGF-I-stimulated cells. The absence of a sub-G0 peak in cell cycle analysis, and the results of the Annexin-V-FITC assay showed cell death was not occurring, neither by apoptosis nor by necrosis. Rather, the researchers found that the lycopene interfered with IGF-IR signaling by reducing IGF-I-induced IRS-1 phosphorylation but not IRS-1 protein levels. Additionally, lycopene reduced the capacity of AP-1 to bind with its transcriptional regulatory complex. Even though the lycopene appeared to up-regulate the AP-1, the binding capacity was in fact blocked. Lycopene had no effect on the expression of the IGF-IR, or on its affinity to bind IGF-I. Lycopene increased the number of cell surface-associated IGFBPs by about 60%. However, due to cross-reactivity of antibodies, the group was unable to identify which specific IGFBPs had been increased (Karas *et al.*, 2000). The authors note the doses of lycopene used are physiologically achievable, as subjects who eat average-to-high amounts of tomato-based foods had similar plasma concentrations of lycopene (<1  $\mu$ M) (Gerster, 1997).

Resveratrol, at doses less than 50  $\mu$ M, was shown to induce S-phase cell cycle arrest followed by apoptotic death in MCF-7 cells (Pozo-Guisado *et al.*, 2002). In MDA-MB-231 cells, however, doses of up to 200  $\mu$ M resveratrol had no effect on cell

cycle and induced non-apoptotic cell death. The same group went on to investigate the role of ER $\alpha$  in resveratrol's effects on cell cycle and apoptosis. The ER $\alpha$  was found to interact with the p85 regulatory subunit of PI3K in vascular endothelial cells (Simoncini *et al.*, 2000) and the same was found to be true of MCF-7 cells (Pozo-Guisado *et al.*, 2004). In the MCF-7 cells, regardless of the presence or absence of estradiol, resveratrol increased ER $\alpha$ -associated PI3K activity at a low dose (10  $\mu$ M) and then proceeded to inhibit the kinase activity in a dose-dependent manner (50 to 150  $\mu$ M). The resveratrol inhibited the kinase activity by inducing proteasome-dependent degradation of the ER $\alpha$ , while levels of p85 were unaffected. In addition, any ER $\alpha$  present continued to bind with the p85. Therefore, the resveratrol did not inhibit binding of these two proteins. In response to resveratrol treatment, Akt phosphorylation followed the same biphasic pattern as the PI3K activity: increased at 10  $\mu$ M, and significantly decreased in response to 50 and 100  $\mu$ M resveratrol. Total expression of Akt remained unchanged. The researchers measured the effects of resveratrol on the phosphorylation status of GSK-3 as well. Again following the same pattern, 10  $\mu$ M resveratrol increased phosphorylation (and thus inactivation) of GSK-3 about 2-fold, while doses higher than 50  $\mu$ M decreased phosphorylation to basal levels. To test whether resveratrol affected the nuclear transcriptional activities of ER $\alpha$ , the researchers measured mRNA levels of two target genes of ER $\alpha$ : Cat-D and pS2. Resveratrol treatment down-regulated the transcription of these two target genes in a dose-dependent manner. In summary, resveratrol exerted estrogenic functions at low concentrations and anti-estrogenic functions at high concentrations in the non-nuclear compartment, and acted only as an estrogen antagonist against ER $\alpha$ 's nuclear transcriptional functions (Pozo-Guisado *et al.*, 2004).

The same group went on to investigate the apoptotic pathways involved in resveratrol-induced apoptosis of MCF-7 cells (Pozo-Guisado *et al.*, 2005). Resveratrol

(10-150  $\mu$ M) down-regulated Bcl-2, which is transcriptionally regulated by NF- $\kappa$ B (Yamamoto and Gaynor, 2004). The resveratrol did in fact modify the expression of the p65/p50 heterodimer of NF- $\kappa$ B in the same biphasic pattern seen in the previous paper: Low doses of resveratrol (10 and 50  $\mu$ M) increased expression of the heterodimer, while higher doses (100 and 150  $\mu$ M) virtually completely blocked expression. The PI3K inhibitor LY294002 interfered with DNA binding of the p65/p50 complex in the presence of 50  $\mu$ M resveratrol, implicating the PI3K pathway in resveratrol's effects on NF- $\kappa$ B and Bcl-2. Resveratrol decreased the amount of p65 protein in the nucleus of the cells while total expression within the cell remained the same. The researchers found the resveratrol increased I- $\kappa$ B expression in the cytosol, which binds and sequesters the p65. Furthermore, high doses of resveratrol inhibited calpain's capacity to degrade I- $\kappa$ B. Use of inhibitors showed this function of resveratrol was mediated by both PI3K and ER $\alpha$ , and possibly by the ER $\alpha$ -dependent PI3K activity described in the previous paper. The apoptosis induced by resveratrol in these MCF-7 cells involved neither caspase-3 nor caspase-8. Rather, the decrease in Bcl-2 decreased the mitochondrial membrane potential, causing an increase in ROS and thus apoptosis ensued. An increase in NO was also observed, which may cause apoptosis or necrosis depending on the redox status of the cell. Since both apoptotic and necrotic cell death were observed in these experiments, the authors predict the levels of ROS and NO may play a role in determining which type of cell death the resveratrol causes in the MCF-7 cells. NF- $\kappa$ B is also responsible for the transcriptional regulation of MMP-9 (matrix-metalloproteinase-9), which is induced by IGF-I. 100  $\mu$ M resveratrol blocked the IGF-I-induced MMP-9 activity, but did not affect MMP-2 activity (10 ng/mL IGF-I). Similarly, resveratrol blocked IGF-I-induced cell migration (10 ng/mL IGF-I; 150  $\mu$ M resveratrol, 40% inhibition).

Vyas *et al.* (2005) investigated the effects of resveratrol on regulation of IGF-II in MCF-7 cells. Similar to the studies discussed above, a biphasic pattern was observed. At a low concentration of resveratrol ( $10^{-6}$  M), IGF-II mRNA and protein expression were increased, as were cell numbers according to BrdU incorporation and the MTT assay. A higher dose of resveratrol ( $10^{-4}$  M) showed the opposite effects, down-regulating IGF-II mRNA and protein, and inhibiting cell proliferation. IGF-I expression was detected neither in the controls nor in the resveratrol-treated MCF-7 cells. Addition of  $17\beta$ -estradiol (E2) also induced IGF-II protein expression, while tamoxifen or high resveratrol did not. Combination treatments of low resveratrol and E2, tamoxifen and E2, low resveratrol and tamoxifen, and high resveratrol and E2 each down-regulated IGF-II expression compared to the induction by low resveratrol or that by E2. Resveratrol appeared to exert mitogenic effects at low concentrations and anti-mitogenic effects at high concentrations. Also, the low dose of resveratrol was mitogenic in the absence of E2 to an extent similar to the E2 alone. However, in the presence of the E2, the resveratrol demonstrated anti-mitogenic effects. Addition of IGF-II rescued the cells from the anti-proliferative effects of the high dose of resveratrol. Also, blocking IGF-IR function with an antibody inhibited the ability of the low dose of resveratrol to stimulate cell proliferation. Together these two pieces of evidence implicate the IGF-II/IGF-IR pathway in resveratrol's mechanisms of action. In similar experiments using the ER-negative MCF10 cell line, the researchers concluded the ability of resveratrol to stimulate proliferation at the low dose is ER-dependent, while the inhibitory actions at the higher dose are ER-independent (Vyas *et al.*, 2005).

Later, the same group demonstrated that in ER(+) MCF-7 (but not ER-) cells, the low dose of resveratrol ( $10^{-6}$  M) increased mRNA and protein expression of cathepsin D, while the high dose ( $10^{-4}$ ) decreased the expression (Vyas *et al.*, 2006).

Cathepsin D is an aspartyl protease responsible for degradation of the extracellular matrix as well as IGFBPs, and is regulated by estrogen and IGF-II (Rochefort *et al.*, 1989; Conover and De Leon, 1994; Claussen *et al.*, 1997; Vyas *et al.*, 2006). The authors recall resveratrol's inability, at both the low and high dose, to increase IGF-II in the presence of E2 or tamoxifen from the prior study, thus noting the potential significance of resveratrol in treating breast cancers. In other words, in the presence of estrogen or tamoxifen, a sufficiently high dose of resveratrol (i.e.  $10^{-4}$  M) may inhibit ER+ breast cancer cell growth via the dual mechanism of blocking expression of the mitogen IGF-II and the protease cathepsin D (Vyas *et al.*, 2006).

In 1992, quercetin was found to be an inhibitor of PI3K using cell-free biochemical extracts. Matter *et al.* (1992) isolated PI3K from bovine brain tissue and measured effects of quercetin and various analogs on kinase activity. Quercetin was the most potent of all compounds tested with an  $IC_{50}$  of 1.3  $\mu\text{g/mL}$  (3.8  $\mu\text{M}$ ). The analog LY805921, which differed from the structure of quercetin only in that it contained an  $\text{OCH}_3$  group rather than an OH at C7, had an  $IC_{50}$  of 1.7  $\mu\text{g/mL}$ . Two other analogs with inhibitory activity had  $IC_{50}$ s of 6.2 and 8.4. Four analogs with low activity had  $IC_{50}$ s  $> 30 \mu\text{g/mL}$ , and another 4 had no activity at all. After structure-activity analysis, the authors found that the substituents of C2 and C3 are critical in determining a compound's ability to inhibit the kinase activity of PI3K. On C3, a hydroxyl group (OH) was found on compounds with potent inhibitory activity ( $IC_{50} < 10 \mu\text{g/mL}$ ); a charged functional group such as a carboxylate or amine abolished inhibitory activity; a bulky substitution increased the  $IC_{50}$ ; and compounds with an H had poorer activity than those with an OH. Where there was an aromatic ring on C2, those compounds with no free hydroxyl groups were poorer inhibitors. An interesting example was genistein, which has a phenol on C3 and no aromatic ring on C2 but an H. Genistein is known as a potent tyrosine kinase inhibitor and protein kinase

inhibitor, yet had no significant activity against PI3K ( $IC_{50} > 30 \mu\text{g/mL}$ ). The authors found quercetin to be a competitive inhibitor of ATP for the ATP binding site on PI3K, with a  $K_i$  of  $0.88 \mu\text{M}$ . Quercetin did not compete against PI (phosphatidylinositol) (Matter *et al.*, 1992).

Agullo *et al.* (1997) also investigated the structure-activity relationship of various flavonoids and their abilities to inhibit PI3K, tyrosine kinase, and protein kinase C (PKC). Out of the flavonoids tested, myricetin, luteolin, quercetin and apigenin were the most potent inhibitors of PI3K in a cell-free biochemical assay, with  $IC_{50}$  values of 1.8, 8, 10 and  $12 \mu\text{M}$ , respectively. The  $IC_{50}$  value for quercetin was higher than that determined by Matter *et al.* (1992), but Agullo *et al.* attribute this to differences in source and purification protocols of the PI3K, and different assay conditions. Comparison among the 14 compounds tested revealed the double bond between C2 and C3 of the flavonoid ring, lack of substitutions on hydroxyl groups, and 3' and 4' hydroxyl groups were the most critical for PI3K inhibitory activity. Presence or absence of OH on the 5C of the A ring did not affect PI3K inhibitory activity. The authors discuss the differences in specificity among the compounds. For example, genistein is specific for inhibition of tyrosine kinases but had no activity against PI3K, while quercetin has inhibitory activity against serine/threonine and lipid kinases as well as against PI3K (Agullo *et al.*, 1997).

Three phytochemicals, genistein, resveratrol and quercetin, were shown to inhibit proliferation and cause apoptosis in MCF-7 cells by altering PTEN expression (Waite *et al.*, 2005). PTEN is a phosphatase that removes the phosphate group from the 3 position of the inositol ring of phosphatidylinositol molecules, thus turning off the signal that PI3K activates by phosphorylating that 3 position (Waite and Eng, 2002). Specifically, quercetin in doses of 0.1 to 1000 nM, increased PTEN protein (and to a lesser extent mRNA expression), decreased Akt phosphorylation, and



increased p27 expression, in keeping with the known lipid phosphatase activity of PTEN. The increase in PTEN expression seen in response to the phytochemical treatment was not due to changes in PTEN stability (as there were no changes in phosphorylation of key residues that determine PTEN stability: Ser<sup>380</sup>, Thr<sup>382</sup> and Thr<sup>383</sup>) or to changes in degradation (as there were no changes in PTEN association with ubiquitin conjugating enzymes UbCH7 and Ubc9). Cyclin D1 expression and MAPK phosphorylation, which are downstream targets of PTEN's protein phosphatase activity, were unaffected by phytochemical treatment (Waite *et al.*, 2005).

Lin *et al.* (2007) demonstrated that IGF-I and estrogen enhance one another's cell proliferative activity in MCF-7 cells, through a ROS-dependent pathway which results in phosphorylation of IRS-1, ERKs and JNKs. After 3 days of incubation, 10 nM E2 nearly doubled MCF-7 cell proliferation, and 10 ng/mL IGF-I in addition to the E2 increased the cell proliferation even further. IGF-I and E2 also individually stimulated colony formation in soft agar, but the effect of the two combined was even greater. In E2/IGF-I treated cells, IRS-1, ERKs and JNKs were activated/phosphorylated, while p38 was not. E2 alone was able to stimulate the ERKs, but the IRS-1 and JNKs were only phosphorylated in the presence of IGF-I. IGF-I magnified E2's induction of c-Jun expression. The E2/IGF-I treatment stimulated H<sub>2</sub>O<sub>2</sub> production in the MCF-7 cells, while preincubation with the antioxidant N-acetyl-L-cysteine (NAC) suppressed E2/IGF-I's capacity to induce IRS-1, ERK and JNK phosphorylation. The aglycones quercetin and baicalein inhibited the E2/IGF-I-induced proliferation, while their respective glycosides, quercitrin (quercetin-3-*O*-rhamnoside), rutin (quercetin-3-*O*-rutinoside), and baicalin (baicalein-7-*O*-glucuronide), did not. The authors attribute this finding to the aglycone's greater antioxidant capacity compared to their respective glycosides (Plumb *et al.*, 1999). In addition, 3-OH flavones also inhibited the actions of E2/IGF-I,

while a compound in which the OH was replaced with an OCH<sub>3</sub> group could not (Lin *et al.*, 2007). The hydroxyl groups of the flavonoids confer antioxidant activity (Cao *et al.*, 1997; Babu *et al.*, 2003), and the authors previously showed that hydroxylation of the C4' and C6 of the flavonoid confers apoptosis-inducing ability (Ko *et al.*, 2004).

## **VI. OBJECTIVES**

The objectives of this research were: 1. To develop a more efficient methylene blue assay for accurate and versatile cell counting; 2. To elucidate the mechanisms of action by which quercetin-3- $\beta$ -D-glucoside (Q-3-G) potentially inhibited IGF-I-induced MCF-7 cell proliferation; and 3. To determine the effects of apple phytochemical extracts on cell proliferation and apoptosis in a DMBA-induced rat mammary cancer model *in vivo*, and to determine how apple phytochemical extracts affect the expression of cell cycle proteins *in vivo*.

## **VII. HYPOTHESIS**

As discussed in this review, cancer risk is highly influenced by diet. Our lab focuses on determining the mechanisms of action by which dietary phytochemicals prevent or slow the growth of cancer cells *in vitro* and *in vivo*. Our hypothesis is apple phytochemical extracts have potent anticancer activity for prevention of breast cancer in humans and could modulate multiple signaling pathways. We also hypothesized that Q-3-G inhibited IGF-I-induced MCF-7 cell proliferation by interfering with IGF-IR signaling pathways, mainly the components of the PI3K pathway, which converge on cell cycle progression and apoptosis suppression. We also hypothesized that feeding whole apple extracts to DMBA-treated Sprague-Dawley rats would modulate early stages of mammary cancer promotion and progression by affecting proliferation and apoptosis *in vivo*.

## REFERENCES

- Adam, C.L. and P.A. Findlay. 1997. Effect of nutrition on testicular growth and plasma concentrations of gonadotrophins, testosterone and insulin-like growth factor I (IGF-I) in pubertal male Soay sheep. *Journal of Reproduction and Fertility*. 111: 121-125.
- Adamo, M.L., L. Wang, L. Heron, D. Ben-Yosef, H. Zhao, and D. Le Roith. 2005. Overview and Molecular Aspects of the Insulin-Like Growth Factor System. In: *IGF and Nutrition in Health and Disease* (M.S. Houston, J.M.P. Holly and E.L. Feldman, editors), Humana Press, Totowa, NJ, 3-22.
- Agullo, G., L. Gamet-Payraastre, S. Manenti, C. Viala, C. Remesy, H. Chap and B. Payraastre. 1997. Relationship between flavonoid structure and inhibition of phosphatidylinositol 3-kinase: a comparison with tyrosine kinase and protein kinase C inhibition. *Biochemical Pharmacology*. 53: 1649-1657.
- Alessi, D.R., M. Andjelkovic, B. Caudwell, P. Cron, N. Morrice, P. Cohen, and B.A. Hemmings. 1996. Mechanism of activation of protein kinase B by insulin and IGF-I. *EMBO Journal*. 15(23): 6541-6551.
- American Cancer Society. 2008. *Cancer Facts & Figures 2008*. American Cancer Society, Atlanta.
- Ames, B.N. and L.S. Gold. 1991. Endogenous mutagens and the causes of aging and cancer. *Mutation Research*. 250: 3-16.
- Ames, B.N., M.K. Shigenaga and L.S. Gold. 1993. DNA lesions, inducible DNA repair, and cell division: three key factors in mutagenesis and carcinogenesis. *Environmental Health Perspectives*. 101(Suppl 5): 35-44.
- Andlauer, W., J. Kolb and P. Furst. 2004. Phloridzin improves absorption of genistin in isolated rat small intestine. *Clinical Nutrition*. 23(5): 989-995.
- Anto, R.J., K. Sukumaran, G. Kuttan, M.N.A. Rao, V. Subbaraju and R. Kuttan. 1995. Anticancer and antioxidant activity of synthetic chalcones and related compounds. *Cancer Letters*. 97(1): 33-37.

- Arai, Y., N. Hirose, K. Yamamura, K. Shimizu, M. Takayama, Y. Ebihara and Y. Osono. 2001. Serum insulin-like growth factor-1 in centenarians: implications of IGF-1 as a rapid turnover protein. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*. 56: M79-M82.
- Arts, I.C., P.C. Hollman, H.B. Bueno De Mesquite, E.J. Feskens and D. Kromhout. 2001. Dietary catechins and epithelial cancer incidence: the Zutphen Elderly Study. *International Journal of Cancer*. 92: 298-302.
- Arts, I.C.W., A.L.A. Sesink and P.C.H. Hollman. 2002. Letter to the Editor: Quercetin-3-glucoside is transported by the glucose carrier SGLT1 across the brush border membrane of rat small intestine. *Journal of Nutrition*. 132: 2823.
- Avila, M.A., J.A. Velasco, J. Cansado and V. Votario. 1994. Quercetin mediates the down-regulation of mutant p53 in the human breast cancer cell line MDA-MB468. *Cancer Research* 54: 2424-2428.
- Aziz, A.A., C.A. Edwards, M.E.J. Lean and A. Crozier. 1998. Absorption and excretion of conjugated flavonols, including quercetin-4'-)-beta-glucoside and isorhamnetin-4'-)-beta-glucoside by human volunteers after the consumption of onions. *Free Radical Research* 29(3): 257-269.
- Babu, B.H., H.N. Jayram, M.G. Nair, K.B. Ajaikumar and J. Padikkala. 2003. Free radical scavenging antitumor and anticarcinogenic activity of gossypin. *Journal of Experimental and Clinical Cancer Research*. 22: 581-589.
- Bakker, B.M., M.C. Welsh, B.H. Ter Kuile, F.I.C. Mensonides, P.A.M. Michels, F.R. Opperdoes and H.W. Westerhoff. 1999. Contribution of glucose transport to the control of the glycolytic flux in *Trypanosoma brucei*. *Proceedings of the National Academy of Science.s* 96: 10098-10103.
- Bartek, J. and J. Lukas. 2001. Pathways governing G1/S transition and their response to DNA damage. *FEBS Letters*. 490: 117-122.
- Baserga, R., F. Peruzzi and K. Reiss. 2003. Mini Review: The IGF-I Receptor in Cancer Biology. *International Journal of Cancer*. 107: 873-877.

- Berrino, F., A. Villarini, M. De Petris, M. Raimondi and P. Rasanisi. 2006. Adjuvant diet to improve hormonal and metabolic factors affecting breast cancer prognosis. *Annals of the New York Academy of Sciences*. 1089: 110-118.
- Bhala, A., M.D. Harris, S. Zirin, L. Corcoran and P. Cohen. 1998. Insulin-like growth factor axis parameters in sick hospitalized neonates. *Journal of Pediatric Endocrinology and Metabolism*. 11: 451-459.
- Bichell, D.P., K. Kikuchi and P. Rotwein. 1992. Growth hormone rapidly activates insulin-like growth factor I gene transcription in vivo. *Molecular Endocrinology*. 6: 1899-1908.
- Blanco, C.L. and R.J. Ferry. 2005. The Insulin-Like Growth Factor Axis in the Fetus and Neonate. In: *IGF and Nutrition in Health and Disease* (M.S. Houston, J.M.P. Holly and E.L. Feldman, editors), Humana Press, Totowa, NJ, 133-143.
- Block, G., B. Patterson and A. Subar. 1992. Fruit, vegetables and cancer prevention: a review of the epidemiological evidence. *Nutrition and Cancer*. 18(1): 1-29.
- Bonjour, J.P., J. Caverzasio and R. Rizzoli. 1991. Homeostasis of inorganic phosphate and the kidney. Vol. 21. In: *Rickets, Nestle Nutrition Workshop Series* (F.H. Glorieux, editor) Raven Press, NY, 35-46.
- Bonjour, J.P., P. Ammann, T. Chevalley and R. Rizzoli. 2005. Nutrition and Insulin Growth Factor-I in Relation to Bone Health and Disease. In: *IGF and Nutrition in Health and Disease* (M.S. Houston, J.M.P. Holly and E.L. Feldman, editors), Humana Press, Totowa, NJ, 177-192.
- Bosetti, C., L. Spertini, M. Parpinel, P. Gnagnarella, P. Lagiou, E. Negri, S. Franceschi, M. Montella, J. Peteraon, J. Dwyer, A. Giacosa and C. LaVecchia. 2005. Flavonoids and breast cancer risk in Italy. *Cancer Epidemiology, Biomarkers & Prevention*. 14(4): 805-808.
- Boyer, J. and R.H. Liu. 2004. Apple phytochemicals and their health benefits. *Nutrition Journal*. 3(5).
- Bunone, G., P.A. Briand, R.J. Miksicek and D. Picard. 1996. Activation of the unliganded estrogen receptor by EGF involves the MAP kinase pathway and direct phosphorylation. *EMBO*. 15: 2174-2183.

- Byrne, C., G.A. Colditz, W.C. Willett, F.E. Speizer, M. Pollak and S.E. Hankinson. 2000. Plasma insulin-like growth factor (IGF) I, IGF-binding protein 3, and mammographic density. *Cancer Research*. 60: 3744-3748.
- Calliste, C.A., J.C. LeBail, P. Trouillas, C. Pouget, G. Habrioux, A.J. Chulia and J.L. Duroux. 2001. Chalcones: structural requirements for antioxidant, estrogenic and antiproliferative activities. *Anticancer Research*. 21(6A): 3949-3956.
- Cantley, L.C. 2002. The Phosphoinositide 3-kinase Pathway. *Science*. 296: 1655-1657.
- Cao, G., E. Sofic and R.L. Prior. 1997. Antioxidant and prooxidant behavior of flavonoids: structure-activity relationships. *Free Radical Biology and Medicine* 22: 749-760.
- Cao, X., L. Fang, S. Gibbs, Y. Huang, Z. Dai, P. Wen, X. Zheng, W. Sadee and D. Sun. 2006. Glucose uptake inhibitor sensitizes cancer cells to daunorubicin and overcomes drug resistance in hypoxia. *Cancer Chemotherapy and Pharmacology*. 59(4): 495-505.
- Cardone, M.H., N. Roy, H.R. Stennicke, G.S. Salvesen, T.F. Franke, E. Stanbridge, S. Grisch and J.C. Reed. 1998. Regulation of cell death protease caspase-9 by phosphorylation. *Science*. 282: 1318-1321.
- Cascio, S., V. Bartella, C. Garofalo, A. Russo, A. Giordano and E. Surmacz. 2007. Insulin-like growth factor 1 differentially regulates estrogen receptor-dependent transcription at estrogen response element and AP-1 sites in breast cancer cells. *The Journal of Biological Chemistry*. 282: 3498-3506.
- Caverzasio, J. and J.P. Bonjour. 1991. IGF-I, a key regulator of renal phosphate transport and 1,25-dihydroxyvitamine D3 production during growth. *News in Physiological Sciences*. 6: 206-210.
- Choi, E.J., S.M. Bae and W.S. Ahn. 2008. Antiproliferative effects of quercetin through cell cycle arrest and apoptosis in human breast cancer MDA-MB-453 cells. *Archives of Pharmacal Research*. 31(10): 1281-1285.
- Choi, J.A., J.Y. Kim, N.Y. Lee, C.M. Kang, H.J. Kwon, Y.D. Yoo, T.W. Kim, Y.S. Lee and S.J. Lee. 2001. Induction of cell cycle arrest and apoptosis in human

breast cancer cells by quercetin. *International Journal of Oncology*. 19(4): 837-844.

Clemmons, D.R., A. Klibanski, L.E. Underwood, J.W. McArthur, E.C. Ridgway, I.Z. Beitins, et al. 1981. Reduction of plasma immunoreactive somatomedin-C during fasting in humans. *Journal of Clinical Endocrinology and Metabolism*. 53: 1247-1250.

COMA. 1998. Nutritional Aspects of the Development of Cancer (Report of the Working Group on Diet and Cancer of the Committee on Medical Aspects of Food and Nutrition Policy). The Stationery Office, London.

Crespy, V., C. Mornad, C. Besson, N. Cotelle, H. Vezin, C. Demigne and C. Remesy. 2003. The splanchnic metabolism of flavonoids highly differed according to the nature of the compound. *AJP-Gastrointestinal and Liver Physiology*. 284: 980-988.

Day, A., J. Gee, M. Dupont, I. Johnson and G. Williamson. 2003. Absorption of quercetin-3-glucoside and quercetin-4'-glucoside in the rat small intestine: the role of lactase phlorizin hydrolase and the sodium dependent glucose transporter. *Biochemical Pharmacology*. 65: 1199-1206.

Day, A.J., M.S. Dupont, S. Ridley, M. Rhodes, M.J.C. Rhodes, M.R.A. Morgan and G. Williamson. 1998. Deglycosylation of flavonoid and isoflavonoid glycosides by human small intestine and liver beta-glucosidase activity. *FEBS Letters*. 436: 71-75.

Day, A.J., F.J. Canada, J.C. Diaz, P.A. Kroon, R. Mclauchlan, C.B. Faulds, G.F. Plumb, M.R.A. Morgan and G. Williamson. 2000. Dietary flavonoid and isoflavone glycosides are hydrolyzed by the lactase site of lactase phlorizin hydrolase. *FEBS Letters*. 468: 166-170.

Delmi, M., C.H. Rapin, J.M. Bengoa, P.D. Delmas, H. Vasey and J.P. Bonjour. 1990. Dietary supplementation in elderly patients with fractured neck of the femur. *Lancet*. 335: 1013-1016.

DeStephani, E., P. Boffetta, H. Deneo-Pelligrini, M. Mendilaharsu, J.C. Carzoglio, et al. 1999. Dietary antioxidants and lung cancer risk: a case-control study in Uruguay. *Nutritional Cancer*. 34: 100-110.

- DeVries, J.H., P.C. Hollman, S. Meyboom, M.N. Buysman, P.L. Zock, *et al.* 1998. Plasma concentrations and urinary excretion of the antioxidant flavonols quercetin and kaempferol as biomarkers for dietary intake. *American Journal of Clinical Nutrition*. 68: 60-65.
- DiPietro, P.F., N.I. Medeiros, F.G. Vieira, M.A. Fausto and A. Bello-Klein. 2007. Breast cancer in southern Brazil: association with past intake. *Nutricion Hospitalaria: Organo Oficial de la Sociedad Espanola de Nutricion Parenteral y Enteral*. 22(5): 565-572.
- Doll, R. and A.B. Hill. 1950. Smoking and carcinoma of the lung. Preliminary report. *British Medical Journal*. ii: 739-748.
- Doll, R. and R. Peto. 1981. The causes of cancer: quantitative estimates of avoidable risks of cancer in the United States today. *Journal of the National Cancer Institute*. 66: 1191-1308.
- Doll, R. and R. Peto. 2003. In: *Oxford Textbook of Medicine* (D.A. Warrell, T.M. Cox and J.D. Firth, editors), Oxford University Press, Oxford.
- Donovan, S.M., R.L. Hintz and R.G. Rosenfeld. 1991. Insulin-like growth factors I and II and their binding proteins in human milk: effect of heat treatment on IGF and IGF binding protein stability. *Journal of Pediatric Gastroenterology and Nutrition*. 13: 242-253.
- Dos Santos Silva, I., P. Mangtani, V. McCormack, D. Bhakta, L. Sevak and A.J. McMichael. 2002. Lifelong vegetarianism and risk of breast cancer: a population-based case-control study among South Asian migrant women living in England. *International Journal of Cancer*. 99: 238-244.
- Downward, J. 1999. How Bad phosphorylation is good for survival. *Nature Cell Biology*. 1: E33-35.
- Dubik, D. and R.P. Shiu. 1992. Mechanism of estrogen activation of c-myc oncogene expression. *Oncogene*. 7: 1587-1594.
- Duffy, M.J., T.M. Maguire, A. Hill, E. McDermott and N. O'Higgins. 2000. Metalloproteinases: role in breast carcinogenesis, invasion and metastasis. *Breast Cancer Research*. 2: 252-257.



- Dufourny, B., J. Alblas, H.A.A.M. van Teeffelen, F.M.A. van Schaik, B. vander Burg, P.H. Steenbergh and J.S. Sussenbach. 1997. Mitogenic signaling of insulin-like growth factor I in MCF-7 human breast cancer cells requires phosphatidylinositol 3-kinase and is independent of mitogen-activated protein kinase. *The Journal of Biological Chemistry*. 272: 31163-31171.
- Duncan, A.M. 2004. The role of nutrition in the prevention of breast cancer. *AACN Clinical Issues*. 15(1): 119-135.
- Dunn, J.E. 1977. Breast cancer among American Japanese in the San Francisco Bay area. *National Cancer Institute Monograph*. 32: 73-79.
- Dunn, S.E., F.W. Kari, J. French, *et al.* 1997. Dietary restriction reduces insulin-like growth factor I levels, which modulates apoptosis, cell proliferation, and tumor progression in p53-deficient mice. *Cancer Research*. 57: 4667-4672.
- Eberhardt, M.V., C.Y. Lee and R.H. Liu. 2000. Antioxidant activity of fresh apples. *Nature*. 405: 903-904.
- El-Tanani, M.K. and C.D. Green. 1997. Two separate mechanisms for ligand-independent activation of the estrogen receptor. *Molecular Endocrinology*. 11: 928-937.
- Fagan, D.H. and D. Yee. 2008. Crosstalk between IGF1R and estrogen receptor signaling in breast cancer. *Journal of Mammary Gland Biology and Neoplasia*. 13: 423-429.
- Faria, A., C. Calhau, V. de Freitas and N. Mateus. 2006. Procyanidins as antioxidants and tumor cell growth modulators. *Journal of Agricultural and Food Chemistry*. 54(6): 2392-2397.
- Felice, D.L., J. Sun and R.H. Liu. 2009. A modified methylene blue assay for accurate cell counting. *Journal of Functional Foods*. 1: 109-118.
- Figueroa, J.A., J. Sharma, J.G. Jackson, M.J. McDermott, S.G. Hilsenbeck and D. Yee. 1993a. Recombinant insulin-like growth factor binding protein-1 inhibits IGF-I, serum, and estrogen-dependent growth of MCF-7 human breast cancer cells. *Journal of Cellular Physiology*. 157: 229-236.

- Figuerola, J.A., J.G. Jackson, W.L. McGuire, R.F. Krywicki and D. Yee. 1993b. Expression of insulin-like growth factor binding proteins in human breast cancer correlates with estrogen receptor status. *Journal of Cellular Biochemistry*. 52: 196-205.
- Forman, M.R. 2007. Changes in dietary fat and fiber and serum hormone concentrations: nutritional strategies for breast cancer prevention over the life course. *The Journal of Nutrition*. 137: 170S-174S.
- Freiss, G., H. Rochefort and F. Vignon. 1990. Mechanisms of 4-hydroxytamoxifen anti-growth factor activity in breast cancer cells: alterations of growth factor receptor binding sites and tyrosine kinase activity. *Biochemical and Biophysical Research Communications*. 173: 919-926.
- Friedman, M., B.E. Mackey, H.J. Kim, I.S. Lee, K.R. Lee, S.U. Lee, E. Kozukue and N. Kozukue. 2007. Structure-activity relationships of tea compounds against human cancer cells. *Journal of Agricultural and Food Chemistry*. 55(2): 243-253.
- Gallus, S., R. Talamini, A. Giacosa, M. Montella, V. Ramazzotti, S. Franceschi, E. Negro and C. LaVecchia. 2005. Does an apple a day keep the oncologist away? *Annals of Oncology*. 16: 1841-1844.
- Gandini, S., H. Merzenich, C. Robertson and P. Boyle. 2000. Meta-analysis of studies on breast cancer risk and diet: the role of fruit and vegetable consumption and the intake of associated micronutrients. *European Journal of Cancer*. 36(5): 636-646.
- Gee, J.M., M.S. DuPont, M.J.C. Rhodes and I.T. Johnson. 1998. Quercetin glucosides interact with the intestinal glucose transport pathway. *Free Radical Biology and Medicine*. 25: 19-25.
- Geier, A., C. Weiss, R. Barry, M. Haimsohn, R. Hemi, Z. Malik and A. Karadik. 1995. Multiple pathways are involved in protection of MCF-7 cells against death due to protein synthesis inhibition. *Journal of Cellular Physiology*. 163(3): 570-576.
- Gerster, H. 1997. The potential role of lycopene for human health. *Journal of the American College of Nutrition*. 16: 109-126.

- Glade, M.J. 1997. Food, nutrition, and the prevention of cancer: a global perspective. American Institute for Cancer Research/World Cancer Research Fund, American Institute for Cancer Research. *Nutrition*. 15(6): 523-526.
- Go, M.L., X. Wu and X.L. Liu. 2005. Chalcones: An update on cytotoxic and chemoprotective properties. *Current Medicinal Chemistry*. 12: 483-499.
- Goldbohm, R.A., P.A. van den Brandt, M.G.L. Hertog, H.A.M. Brants and G. van Poppel. 1995. Flavonoid intake and risk of cancer: a prospective cohort study. *American Journal of Epidemiology*. 41: s61.
- Goodin, M.G., K.C. Fertuck, T.R. Zacharewski and R.J. Rosengren. 2002. Estrogen Receptor-mediated actions of polyphenolic catechins in vivo and in vitro. *Toxicological Sciences*. 69: 354-361.
- Graefe, E.U., J. Wittig, S. Mueller, A-K. Riethling, B. Uehleke, B. Drewelow, H. Pforte, G. Jacobasch, H. Derendorf and M. Veit. 2001. Pharmacokinetics and bioavailability of quercetin glycosides in humans. *Journal of Clinical Pharmacology*. 41: 492-499.
- Graf, B.A., W. Mullen, S.T. Caldwell, R.C. Hartley, G.G. Duthie, M.E.J. Lean, A. Crozier and C.A. Edwards. 2005. Disposition and metabolism of [2-<sup>14</sup>C]quercetin-4'-glucoside in rats. *Drug Metabolism and Disposition*. 33: 1036-1043.
- Hackett, A.M. 1986. In: *Plant Flavonoids in Biology and Medicine: Biochemical, Pharmacological and Structure-Activity Relationships* (V. Cody, E. Middleton and J.B. Harborne, editors), Alan Liss, NY, 177-194.
- Hadsell, D.L., K.L. Murphy, S.G. Bonnette, N. Reece, R. Laucirica and J.M. Rosen. 2000. Cooperative interaction between mutant p53 and des(1-3)IGF-I accelerates mammary tumorigenesis. *Oncogene*. 19(7): 889-898.
- Hakimuddin, F., G. Paliyath and K. Meckling. 2004. Selective cytotoxicity of a red grape wine flavonoid fraction against MCF-7 cells. *Breast Cancer Research and Treatment*. 85(1): 65-79.

- Hanf, V. and U. Gonder. 2005. Nutrition and primary prevention of breast cancer: foods, nutrients and breast cancer risk. *European Journal of Obstetrics & Gynecology*. 123: 139-149.
- Hankinson, S.E., W.C. Willett, G.A. Colditz, D.J. Hunter, D.S. Michaud, B. Deroo, B. Rosner, F.E. Speizer and M. Pollak. 1998. Circulating concentrations of insulin-like growth factor-I and risk of breast cancer. *Lancet*. 351(9113): 1393-1396.
- He, X. and R.H. Liu. 2007. Triterpenoids isolated from apple peels have potent antiproliferative activity and may be partially responsible for apple's anticancer activity. *Journal of Agricultural and Food Chemistry*. 55(11): 4366-4370.
- He, X. and R.H. Liu. 2008. Phytochemicals of apple peels: isolation, structure elucidation, and their antiproliferative and antioxidant activities. *Journal of Agricultural and Food Chemistry*. 56(21): 9905-9910.
- Henricks, D.M., A.J. Kouba, B.R. Lackey, W.R. Boone and S.L. Gray. 1998. Identification of insulin-like growth factor I in bovine seminal plasma and its receptor on spermatozoa: influence on sperm motility. *Biology of Reproduction*. 59: 330-337.
- Hertog, M.G., E.J. Feskens, P.C. Hollman, M.B. Katan and D. Kromhout. 1993a. Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study. *The Lancet*. 342: 1007-1011.
- Hertog, M.G.L, P.C.H. Hollman, M.B. Katan, and D. Kromhout. 1993b. Intake of potentially anticarcinogenic flavonoids and their determinants in adults in the Netherlands. *Nutritional Cancer*. 20: 21-29.
- Hertog, M.G.L., D. Kromhout, C. Aravanis, *et al.* 1995. Flavonoid intake and long-term risk of coronary heart disease and cancer in the Seven Countries Study. *Archives of Internal Medicine*. 155: 381-386.
- Hollman, P.C.H., J.H.M. deVries, S.D. vanLeeuwen, M.J.B. Mengelers and M.B. Katan. 1995. Absorption of dietary quercetin glycosides and quercetin in healthy ileostomy volunteers. *American Journal of Clinical Nutrition*. 62: 1276-1282.

- Hollman, P.C.H., J.M.P. van Trijp, N.C.P. Buysmas, M.S. vander Gaag, M.J.B. Mengelers, J.H.M. deVries and M.B. Katan. 1997. Relative bioavailability of the antioxidant flavonoid quercetin from various foods in man. *FEBS Letters*. 418: 152-156.
- Holly, J.M., D.J. Gunnell and G. Davey Smith. 1999. Growth hormone, IGF-I and cancer. Less intervention to avoid cancer? More intervention to prevent cancer? *The Journal of Endocrinology*. 162(3): 321-330.
- Holmes, M.D. and W.C. Willett. 2004. Does diet affect breast cancer risk? *Breast Cancer Research*. 6(4): 170-178.
- Houston, M.S., J.M.P. Holly and E.L. Feldman, editors. 2005. *IGF and Nutrition in Health and Disease*. Humana Press, Totowa, NJ,
- Hung, H. 2003. Inhibition of estrogen receptor alpha expression and function in MCF-7 cells by kaempferol. *Journal of Cellular Physiology*. 198(2): 197-208.
- Huynh, H., X. Yang and M. Pollak. 1996. Estradiol and antiestrogens regulate a growth inhibitory insulin-like growth factor binding protein 3 autocrine loop in human breast cancer cells. *Journal of Biological Chemistry*. 271: 1016-1021.
- Hynes, N.E. and H.A. Lane. 2005. ERBB receptors and cancer: The complexity of targeted inhibitors. *Nature Reviews Cancer*. 5: 341-354.
- IARC. Schistosomes, Liver Flukes and *Helicobacter pylori*. *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans*, 61. IARC, Lyon, 1994a. IARC. Hepatitis Viruses. *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans*, 59. IARC, Lyon, 1994b.
- Ignar-Trowbridge, D.M., C.T. Teng, K.A. Ross, M.G. Parker, K.S. Korach and J.A. McLachlan. 1993. Peptide growth factors elicit estrogen receptor-dependent transcriptional activation of an estrogen-responsive element. *Molecular Endocrinology*. 7: 992-998.
- Ingram, D.M., A. Roberts and E.M. Nottage. 1992. Host factors and breast cancer growth characteristics. *European Journal of Cancer*. 28A: 1153-1161.

- Ioku, K., Y. Pongpiriyadacha, Y. Konishi, Y. Takei, N. Nakatani and J. Terao. 1998. Beta-glucosidase activity in the rat small intestine toward quercetin monoglucosides. *Bioscience, Biotechnology and Biochemistry*. 62: 1428-1431.
- Isley, W.L., L.E. Underwood and D.R. Clemmons. 1983. Dietary components that regulate serum somatomedin-C concentrations in humans. *The Journal of Clinical Investigation*. 71: 175-182.
- Izumi, T., M.F. White, T. Kadowaki, F. Takaku, Y. Akanuma and M. Kasuga. 1987. Insulin-like growth factor I rapidly stimulates tyrosine phosphorylation of a Mr 185,000 protein in intact cells. *Journal of Biological Chemistry*. 262: 1282-1287.
- Jeong, J.H., J.Y. An, Y.T. Kwon, L.Y. Li and Y.J. Lee. 2008. Quercetin-induced ubiquitination and down-regulation of Her-2/neu. *Journal of Cellular Biochemistry*. 105(2): 585-595.
- Jeong, J.H., J.Y. An, Y.T. Kwon, J.G. Rhee and Y.J. Lee. 2009. Effects of low dose quercetin: Cancer cell-specific inhibition of cell cycle progression. *Journal of Cellular Biochemistry*. 106(1): 73-82.
- Josefson, D. 2001. Obesity and inactivity fuel global cancer epidemic. *British Medical Journal*. 322: 945.
- Karas, M., H. Amir, D. Fishman, M. Danilenko, S. Segal, A. Nahum, A. Koifmann, Y. Giat, J. Levy and Y. Sharoni. 2000. Lycopene interferes with cell cycle progression and insulin-like growth factor I signaling in mammary cancer cells. *Nutrition and Cancer*. 36(1): 101-111.
- Kato, S., H. Endoh, Y. Masuhiro, T. Kitamoto, S. Uchiyama, H. Sasaki, *et al.* 1995. Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. *Science*. 270: 1491-1494.
- Kaytor, E.N., J.L. Zhu, C.I. Pao and L.S. Phillips. 2001. Insulin-responsive nuclear proteins facilitate Ap1 interactions with the insulin-like growth factor-I gene. *Journal of Biological Chemistry*. 276: 36896-36901.

- Kaytor, E.N., J.L. Zhu, C.I. Pao and L.S. Phillips. 2001b. Physiological concentrations of insulin promote binding of nuclear proteins to the insulin-like growth factor I gene. *Endocrinology*. 142: 1041-1049.
- Kellett, G.L. 2001. The facilitated component of intestinal glucose absorption. *Journal of Physiology*. 531: 585-595.
- Kelsey, J.L., M.D. Gammon and E.M. John. 1993. Reproductive factors and breast cancer. *Epidemiologic Reviews*. 15: 36-47.
- Kleinberg, D.L. and W. Ruan. 2008. IGF-I, GH, and sex steroid effects in normal mammary gland development. *Journal of Mammary Gland Biology and Neoplasia*. 13: 353-360.
- Knekt, P., J. Kumpulainen, R. Jarvinen, H. Rissanen, M. Heliovaara, A. Reunanen, T. Hakulinen and A. Aromaa. 2002. Flavonoid intake and risk of chronic diseases. *American Journal of Clinical Nutrition*. 76(3): 560-568.
- Knekt, P., R. Jarvinen, R. Seppanen, M. Heliovaara, L. Teppo, *et al.* 1997. Dietary flavonoids and the risk of lung cancer and other malignant neoplasms. *American Journal of Epidemiology*. 146: 223-230.
- Ko, C.H., S.C. Shen and Y.C. Chen. 2004. Hydroxylation at C4' or C6 is essential for apoptosis-inducing activity of flavanone through activation of the caspase-3 cascade and production of reactive oxygen species. *Free Radical Biology and Medicine*. 36: 897-910.
- Krishnaraj, R., A. Zaks and T. Unterman. 1998. Relationship between plasma IGF-I levels, in vitro correlates of immunity, and human senescence. *Clinical Immunology and Immunopathology*. 88: 264-270.
- Krupka, R.M. 1985. Asymmetrical binding of phloretin to the glucose transport system of human erythrocytes. *Journal of Membrane Biology*. 83: 71-80.
- Lackey, B.R., W.R. Boone, S.L. Gray and D.M. Henricks. 1998. Computer-assisted sperm motion analysis of bovine sperm treated with insulin-like growth factor I and II: implications as motility regulators and chemokinetic factors. *Archives of Andrology*. 41: 115-125.

- Lang, C.H. and R.A. Frost. 2005. Effect of Insulin-Like Growth Factor Proteins on Skeletal Muscle Protein Metabolism During Normal and Catabolic Conditions. In *IGF and Nutrition in Health and Disease* (M.S. Houston, J.M.P. Holly and E.L. Feldman, editors), Humana Press, Totowa, NJ, 193-209.
- Lann, D. and D. LeRoith. 2008. The role of endocrine insulin-like growth factor-I and insulin in breast cancer. *Journal of Mammary Gland Biology and Neoplasia*. 13: 371-379.
- Lawlor, M.A. and D.R. Alessi. 2001. PKB/Akt: a key mediator of cell proliferation, survival and insulin responses? *Journal of Cell Science*. 114: 2903-2910.
- Lee, A.V., P. Darbre and R.J. King. 1994. Processing of insulin-like growth factor-II (IGF-II) by human breast cancer cells. *Molecular and Cellular Endocrinology*. 99: 211-220.
- Lee, A.V., C.N. Weng, J.G. Jackson and D. Yee. 1997. Activation of estrogen receptor-mediated gene transcription by IGF-I in human breast cancer cells. *Journal of Endocrinology*. 152: 39-47.
- Lee, A.V., J.G. Jackson, J.L. Gooch, S.G. Hilsenbeck, E. Coronado-Heinsohn, C.K. Osborne, *et al.* 1999. Enhancement of insulin-like growth factor signaling in human breast cancer: estrogen regulation on insulin receptor substrate-1 expression in vitro and in vivo. *Molecular Endocrinology*. 13: 787-796.
- Leese, H.J. and G. Semanza. 1973. On the identity between the small intestinal enzymes phlorizen hydrolase and glycosylceramidase. *Journal of Biological Chemistry*. 248: 8170-8173.
- Lewitt, M.S. and K. Hall. 2005. The Insulin Growth Factor System and Nutrition in Adulthood and Aging. In: *IGF and Nutrition in Health and Disease* (M.S. Houston, J.M.P. Holly and E.L. Feldman, editors), Humana Press, Totowa, NJ, 157-174.
- Lin, C-W, L-Y Yang, S-C Shen and Y-C Chen. 2007. IGF-I plus E2 induces proliferation via activation of ROS-dependent ERKs and JNKs in human breast carcinoma cells. *Journal of Cellular Physiology*. 212: 666-674.



- Lin, C.W., W.C. Hou, S.C. Shen, S.H. Juan, C.H. Ko, L.M. Wang and Y.C. Chen. 2008. Quercetin inhibition of tumor invasion via suppressing PKCdelta/ERK/AP-1-dependent matrix metalloproteinase-9 activation in breast carcinoma cells. *Carcinogenesis*. 29(9): 1807-1815.
- Liu, J.R., H. Dong, B. Chen, P. Zhao and R.H. Liu. 2009. Fresh apples suppress mammary carcinogenesis, proliferative activity, and induce apoptosis in the mammary tumors of the Sprague-Dawley rat. *Journal of Agricultural and Food Chemistry*. 57(1): 297-304.
- Liu, R.H. and J.H. Hotchkiss. 1995. Potential genotoxicity of chronically elevated nitric oxide: A review. *Mutation Research*. 339: 73-89.
- Liu, R.H. and D.L. Felice. 2007. Antioxidants and Whole Food Phytochemicals for Cancer Prevention. In *Antioxidant Measurement and Applications* (S. Fereidoon and C-T Ho, editors), American Chemical Society, Washington, D.C., 15-34.
- Liu, R.H. and J. Sun. 2003. Antiproliferative activity of apples is not due to phenolic-induced hydrogen peroxide formation. *Journal of Agricultural and Food Chemistry*. 51(6): 1718-1723.
- Liu, R.H. 2003. Health benefits of fruit and vegetables are from additive and synergistic combinations of phytochemicals. *The American Journal of Clinical Nutrition*. 78: 517S-520S.
- Liu, R.H. 2004. Potential synergy of phytochemicals in cancer prevention: mechanism of action. *The Journal of Nutrition*. 134: 3479S-3485S.
- Liu, R.H., J.R. Liu and B. Chen. 2005. Apples prevent mammary tumors in rats. *Journal of Agricultural and Food Chemistry*. 53: 2341-2343.
- Lostao, M.P., B.A. Hirayama, D.D.F. Loo and E.M. Wright. 1994. Phenylglucosides and the Na<sup>+</sup>/glucose cotransporter (SGLT1): Analysis of interactions. *Journal of Membrane Biology*. 142: 161-170.
- Maison, P., B. Balkau, *et al.* 1998. Growth hormone as a risk for premature mortality in healthy subjects: data from the Paris prospective study. *British Medical Journal*. 316: 1132-1133.

- Manach, C., C. Morand, V. Crespy, C. Demigne, O. Texier, F. Regerat and C. Remesy. 1998. Quercetin is recovered in human plasma as conjugated derivatives which retain antioxidant properties. *FEBS Letters*. 426: 331-336.
- Mathieu, M., F. Vignon, F. Capony and H. Rochefort. 1991. Estradiol down-regulates the mannose-6-phosphate/insulin-like growth factor-II receptor gene and induces cathepsin-D in breast cancer cells: a receptor saturation mechanism to increase the secretion of lysosomal proenzymes. *Molecular Endocrinology*. 5: 815-822.
- Matter, W.F., R.F. Brown and C.J. Vlahos. 1992. The inhibition of phosphatidylinositol 3-kinase by quercetin and analogs. *Biochemical and Biophysical Research Communications*. 186(2): 624-631.
- Mawson, A., A. Lai, J.S. Carroll, C.M. Sergio, C.J. Mitchell and B. Sarcevic. 2005. Estrogen and insulin/IGF-I cooperatively stimulate cell cycle progression in MCF-7 breast cancer cells through differential regulation of c-myc and cyclin D1. *Molecular and Cellular Endocrinology*. 229: 161-173.
- McMichael, A.J., M.G. McCall, J.M. Harshorne and T.L. Woodings. 1980. Patterns of gastro-intestinal cancer in European migrants to Australia. The role of dietary change. *International Journal of Cancer*. 25: 431-437.
- Meyer, G., A. Bole and E.L. Feldman. 2005. Insulin-Like Growth Factors and Nervous System Disorders. In: *IGF and Nutrition in Health and Disease* (M.S. Houston, J.M.P. Holly and E.L. Feldman, editors), Humana Press, Totowa, NJ, 211-225.
- Morand, C., V. Crespy, C. Manach, C. Bessen, C. Demigne and C. Remesy. 1998. Plasma metabolites of quercetin and their antioxidant properties. *American Journal of Physiology*. 275: R212-R219.
- Morishita, S., K. Niwa, S. Ichigo, M. Hori, T. Murase, J. Fujimoto, *et al.* 1995. Overexpressions of c-fos/jun mRNA and their oncoproteins (Fos/Jun) in the mouse uterus treated with three natural estrogens. *Cancer Letters*. 97: 225-231.
- Morre, D.J., A. Bridge, L.Y. Wu and D.M. Morre. 2000. Preferential inhibition by (-)-epigallocatechin-3-gallate of the cell surface NADH oxidase and growth transformed cells in culture. *Biochemical Pharmacology*. 60: 937-946.

- Mulder, J.G. and W.B. Jakoby. 1990. Sulfation. In: *Conjugation Reactions in Drug Metabolism* (G.J. Mulder, editor), Taylor and Francis, London, 107-161.
- Musgrove, E.A. and R.L. Sutherland. 1994. Cell cycle control by steroid hormones. *Seminars in Cancer Biology*. 5: 381-389.
- Nakachi, K., K. Suemasu, K. Suga, T. Takeo, K. Imai and Y. Higashi. 1998. Influence of drinking tea on breast cancer malignancy among Japanese patients. *Japanese Journal of Cancer Research*. 89: 254-261.
- Nelson, J.A.S. and R.E. Falk. 1993. Phloridzin and phloretin inhibition of 2-deoxy-D-glucose uptake by tumor cells in vitro and in vivo. *Anticancer Research*. 13: 2293-2299.
- Nickerson, T., H. Huynh and M. Pollak. 1997. Insulin-like growth factor binding protein-3 induces apoptosis in MCF7 breast cancer cells. *Biochemical and Biophysical Research Communications*. 237: 690-693.
- Olayioye, M.A., R.M. Neve, H.A. Lane and N.E. Hynes. 2000. The ErbB signaling network: Receptor heterodimerization in development and cancer. *EMBO Journal*. 19: 3159-3167.
- Older, M.W.J., D. Edwards and J.W.T. Dickerson. 1980. A nutrient survey in elderly women with femoral neck fractures. *British Journal of Surgery*. 67: 884-886.
- Olthof, M.R., P.C.H. Hollman, T.B. Vree and M.B. Katan. 2000. Bioavailabilities of quercetin-3-glucoside and quercetin-4'-glucoside do not differ in humans. *Journal of Nutrition*. 130: 1200-1203.
- Osborne, C.K., E.B. Coronade, L.J. Kitten, C.I. Arteaga, S.A. Fuqua, K. Ramasharma, *et al.* 1989. Insulin-like growth factor-II (IGF-II): a potential autocrine/paracrine growth factor for human breast cancer acting via the IGF-I receptor. *Molecular Endocrinology*. 3: 1701-1709.
- Paolisso, G., S. Ammendola, A. Delbuono, A. Gambardella, M. Riondino, M.R. Tagliamonte, M.R. Rizzo, C. Carella and M. Varricchio. 1997. Serum levels of insulin-like growth factor-I (IGF-I) and IGF-binding protein-3 in healthy centenarians-relationship with plasma leptin and lipid concentrations, insulin

action, and cognitive function. *Journal of Clinical Endocrinology and Metabolism*. 82: 2204-2209.

Peruzzi, F., M. Prisco, M. Dews, P. Salomoni, E. Grassilli, G. Romano, B. Calabretta, and R. Baserga. 1999. Multiple signaling pathways of the Insulin-like growth factor I receptor in protection from apoptosis. *Molecular and Cellular Biology*. 19(10): 7203-7215.

Peterson, J., P. Lagiou, E. Samoli, A. Lagiou, K. Katsouyanni, C. LaVecchia, J. Dwyer, and D. Trichopoulos. 2003. Flavonoid intake and breast cancer risk: a case-control study in Greece. *British Journal of Cancer*. 89: 1255-1259. Peto, J., A. Decarli, C. La Vecchia, F. Levi and E. Negri. 1999. The European mesothelioma epidemic. *British Journal of Cancer*. 79: 666-672.

Peto, J. 2001. Cancer epidemiology in the last century and the next decade. *Nature*. 411: 390-395.

Piskula, M.K. and J. Terao. 1998. Quercetin's solubility affects its accumulation in rat plasma after oral administration. *Journal of Agriculture and Food Chemistry*. 46: 4313-4317.

Plumb, G.W., K.R. Price and G. Williamson. 1999. Antioxidant properties of flavonol glycosides from green beans. *Redox Report*. 4: 123-127.

Pozo-Guisado, E., A. Alvarez-Barrientos, S. Mulero-Navarro, B. Santiago-Josefat and P.M. Fernandez-Salguero. 2002. The antiproliferative activity of resveratrol results in apoptosis in MCF-7 but not in MDA-MB-231 human breast cancer cells: cell-specific alteration of the cell cycle. *Biochemical Pharmacology*. 64: 1375-1386.

Pozo-Guisado, E., M.J. Lorenzo-Benayas and P.M. Fernandez-Salguero. 2004. Resveratrol modulates the phosphoinositide 3-kinase pathway through an estrogen receptor alpha-dependent mechanism: relevance in cell proliferation. *International Journal of Cancer*. 109: 167-173.

Pozo-Guisado, E., J.M. Merino, S. Mulero-Navarro, M.J. Lorenzo-Benayas, F. Centeno, A. Alvarez-Barrientos and P.M. Fernandez-Salguero. 2005. Resveratrol-induced apoptosis in MCF-7 human breast cancer cells involves a caspase-independent mechanism with downregulation of Bcl-2 and NF-kB. *International Journal of Cancer*. 115: 74-84.

- Prosser, C.G. 1996. Insulin-like growth factors in milk and mammary gland. *Journal of Mammary Gland Biology and Neoplasia*. 1: 298-306.
- Rabkin, R., S.S. Guest and F. Schaefer. 2005. The Kidney and the Insulin-Like Growth Factor System in Health and Disease. In: *IGF and Nutrition in Health and Disease* (M.S. Houston, J.M.P. Holly and E.L. Feldman, editors), Humana Press, Totowa, NJ, 227-247.
- Rock, C.L. 2003. Diet and breast cancer: can dietary factors influence survival? *Journal of Mammary Gland Biology and Neoplasia*. 8(1): 119-132.
- Ross, J.A. and C.M. Kasum. 2002. Dietary flavonoids: bioavailability, metabolic effects, and safety. *Annual Review of Nutrition*. 22: 19-34.
- Rowzee, A.M., D.A. Lazzarino, L. Rota, Z. Sun and T.L. Wood. 2008. IFG Ligand and Receptor Regulation of Mammary Development. *Journal of Mammary Gland Biology and Neoplasia*. 13: 361-370.
- Ryan, P.D. and P.E. Goss. 2008. The emerging role of the insulin-like growth factor pathway as a therapeutic target in cancer. *The Oncologist*. 13(1): 16-24.
- Salerno, M., D. Sisci, L. Mauro, M.A. Guvakova, S. Ando and E. Surmacz. 1999. Insulin receptor substrate 1 is a target for the pure antiestrogen ICI 182,780 in breast cancer cells. *International Journal of Cancer*. 81: 299-304.
- Sarbassov, D.D., D.A. Guertin, S.M. Ali and D.M. Sabatini. 2005. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science*. 307: 1098-1101.
- Schlachterman, A., F. Valle, K.M. Wall, N.G. Azios, L. Castillo, L. Morell, A.V. Washington, L.A. Cubano and S.F. Dharmawardhane. 2008. Combined resveratrol, quercetin, and catechin treatment reduces breast tumor growth in a nude mouse model. *Translational Oncology*. 1(1): 19-27.
- Sesink, A.L., K.A. O'Leary and P.C. Hollman. 2001. Quercetin glucuronides but not glucosides are present in human plasma after consumption of quercetin-3-glucoside or quercetin-4'-glucoside. *Journal of Nutrition*. 131(7): 1938-1941.

- Sesink, A.L.A., I.C.W. Arts, M. Faassen-Peters and C.H.H. Hollman. 2003. Intestinal uptake of quercetin-3-glucoside in rats involves hydrolysis by lactase phlorizin hydrolase. *Journal of Nutrition*. 133: 773-776.
- Shemer, J., M. Adamo, G.L. Wilson, D. Heffez, Y. Zick and D. LeRoith. 1987. Insulin and insulin-like growth factor-I stimulate a common endogenous phosphoprotein substrate (pp185) in intact neuroblastoma cells. *Journal of Biological Chemistry*. 262: 15476-15482.
- Simmen, F.A., I. Kwak, J.L. Blum and R.C.M. Simmen. 2005. Nutrition and the IGF System in Reproduction. In: *IGF and Nutrition in Health and Disease* (M.S. Houston, J.M.P. Holly and E.L. Feldman, editors), Humana Press, Totowa, NJ, 145-155.
- Simoncini, T., A. Hafezi-Moghadam, D.P. Brazil, K. Ley, W.W. Chin and J.K. Liao. 2000. Interaction of oestrogen receptor with the regulatory subunit of phosphatidylinositol-3-OH kinase. *Nature*. 407: 538-541.
- Singh, A.V., A.A. Franke, G.L. Blackburn and J-R. Zhao. 2006. Soy phytochemicals prevent orthotopic growth and metastasis of bladder cancer in mice by alterations of cancer cell proliferation and apoptosis and tumor angiogenesis. *Cancer Research*. 66: 1851-1858.
- Slattery, M.L. 2001. Does an apple a day keep breast cancer away? *JAMA*. 285(6): 799-801.
- Smith, W.J., L.E. Underwood, L. Keyes and D.R. Clemmons. 1997. Use of insulin-like growth factor I (IGF-I) and IGF binding protein measurements to monitor feeding of premature infants. *Journal of Clinical Endocrinology and Metabolism*. 82: 3982-3988.
- Smith-Warner, S.A., D. Spiegelman, S-S. Yaun, H-O Adami, W.L. Beeson, P.A. van den Brandt, A.R. Folsom, *et al.* 2001. Intake of fruits and vegetables and risk of breast cancer. A pooled analysis of cohort studies. *The Journal of the American Medical Association*. 285(6): 769-776.
- Song, R.X., C.J. Barnes, Z. Zhang, Y. Bao, R. Kumar and R.J. Santen. 2004. The role of Shc and insulin-like growth factor 1 receptor in mediating the translocation of estrogen receptor alpha to the plasma membrane. *Proceedings of the National Academy of Sciences*. 101(7): 2076-2081.

- Stanford, J.L., L.J. Herrinton, S.M. Schwartz and N.S. Weiss. 1995. Breast cancer incidence in Asian migrants to the United States and their descendants. *Epidemiology*. 6: 181-183.
- Steinmetz, K.A. and J.D. Potter. 1996. Vegetables, fruit and cancer prevention: A review. *Journal of the American Dietetic Association*. 96(10): 1027-1039.
- Stewart, A.J., B.R. Westley and F.E. May. 1992. Modulation of the proliferative response of breast cancer cells to growth factors by oestrogen. *British Journal of Cancer*. 66: 640-648.
- Sun, J. and R.H. Liu. 2008. Apple phytochemical extracts inhibit proliferation of estrogen-dependent and estrogen-independent human breast cancer cells through cell cycle modulation. *Journal of Agricultural and Food Chemistry*. 56: 11661-11667.
- Sun, J., Y. Chu, X. Wu and R.H. Liu. 2002. Antioxidant and antiproliferative activities of common fruits. *Journal of Agricultural and Food Chemistry*. 50: 7449-7454.
- Swanson, S.M. and T.G. Unterman. 2002. The growth hormone-deficient Spontaneous Dwarf rat is resistant to chemically induced mammary carcinogenesis. *Carcinogenesis*. 23(6): 977-982.
- Tang, Y., D.Y. Zhao, S. Elliott, W. Zhao, T.J. Curiel, B.S. Bechman and M.E. Burow. 2007. Epigallocatechin-3 gallate induces growth inhibition and apoptosis in human breast cancer cells through survivin suppression. *International Journal of Oncology*. 31(4): 705-711.
- Terry, P., A. Wolk, I. Persson and C. Magnusson. 2001. Brassica vegetables and breast cancer risk. *JAMA*. 285: 2975-2977.
- Thissen, J.P., J.M. Ketelslegers and L.E. Underwood. 1994. Nutritional regulation of the insulin-like growth factors. *Endocrine Reviews*. 15: 80-101.
- Thissen, J.P., V. Beauloye, J.M. Ketelslegers and L.E. Underwood. 2005. Regulation of Insulin-Like Growth Factor-I by Nutrition. In: *IGF and Nutrition in Health and Disease* (M.S. Houston, J.M.P. Holly and E.L. Feldman, editors), Humana Press, Totowa, NJ, 25-52.

- Thordarson, G., S. Semaan, C. Low, D. Ochoa, H. Leong, L. Rajkumar, *et al.* 2004. Mammary tumorigenesis in growth hormone deficient spontaneous dwarf rats; effects of hormonal treatments. *Breast Cancer Research and Treatment*. 87(3): 277-290.
- Toggenburger, G., M. Kessler and G. Semenza. 1982. Phlorizin as a probe of the small-intestinal Na<sup>+</sup>, D-glucose cotransporter. A model. *Biochimica et Biophysica Acta*. 688: 557-571.
- Tsao, R., R. Yang, J.C. Young and H. Zhu. 2003. Polyphenolic profiles in eight apple cultivars using high-performance liquid chromatography (HPLC). *Journal of Agricultural and Food Chemistry*. 51(21): 6347-6353.
- Vijayababu, M.R., A. Arunkumar, P. Kanagaraj and J. Arunakaran. 2006. Effects of quercetin on insulin-like growth factors (IGFs) and their binding protein-3 (IGFBP3) secretion and induction of apoptosis in human prostate cancer cells. *Journal of Carcinogenesis*. 5(1): 10.
- Voorrips, L.E., R.A. Goldbohm, H.A.M. Brants, A.F.C. Geert, G. van Poppel, F. Sturmans, R.J.J. Hermus. 2000. A prospective cohort study on antioxidant and folate intake and male lung cancer risk. *Cancer Epidemiology, Biomarkers, and Prevention*. 9: 357-365.
- Vyas, S., Y. Asmerom and D.D. De Leon. 2005. Resveratrol regulates insulin-like growth factor-II in breast cancer cells. *Endocrinology*. 146(10): 4224-4233.
- Vyas, S., Y. Asmerom and D.D. De Leon. 2006. Insulin-like growth factor II mediates resveratrol stimulatory effect on cathepsin D in breast cancer cells. *Growth Factors*. 24(1): 79-87.
- Waite, K.A. and C. Eng. 2002. Protean PTEN: form and function. *American Journal of Human Genetics*. 70: B29-B44.
- Waite, K.A., M.R. Sinden and C. Eng. 2005. Phytoestrogen exposure elevates PTEN levels. *Human Molecular Genetics*. 14(11): 1457-1463.
- Wakeling, A.E., E. Newbould and S.W. Peters. 1989. Effects of antioestrogens on the proliferation of MCF-7 human breast cancer cells. *Journal of Molecular Endocrinology*. 2: 225-234.



- Walboomers, J.M., M.V. Jacobs, M. M. Manos, F. X. Bosch, J.A. Kummer, K.V. Shah, P.J.F. Snijders, J. Peto, C.J.L.M. Meijer and N. Munoz. 1999. Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. *Journal of Pathology*. 189: 12-19.
- Walgren, R.A., J-T. Lin, R.K-H. Kinne and T. Walle. 2000. Cellular uptake of dietary flavonoid quercetin 4'-beta-glucoside by sodium-dependent glucose transporter SGLT1. *Pharmacology and Experimental Therapeutics*. 294(3): 837-843.
- Walle, T., Y. Otake, K. Walle and F. Wilson. 2000. Quercetin glucosides are completely hydrolyzed in ileostomy patients before absorption. *Journal of Nutrition*. 130: 2658-2661.
- Walle, T., U.K. Walle and P.V. Halushka. 2001. Carbon dioxide is the major metabolite of quercetin in humans. *Journal of Nutrition*. 131: 2648-2652.
- Wang, S., V.L. DeGroff and S.K. Clinton. 2003. Tomato and soy polyphenols reduce insulin-like growth factor-I-stimulated rat prostate cancer cell proliferation and apoptotic resistance in vitro via inhibition of intracellular signaling pathways involving tyrosine kinase. *Journal of Nutrition*. 133: 2367-2376.
- Werner, H. and D. LeRoith. 2000. New concepts in regulation and function of the insulin-like growth factors: implications for understanding normal growth and neoplasia. *Cell and Molecular Life Sciences*. 57: 932-942.
- Willett, W.C. 1995. Diet, nutrition and avoidable cancer. *Environmental Health Perspectives*. 103(S8): 165-170.
- Williamson, G., A.J. Day, G.W. Plumb and D. Couteau. 2000. Human metabolic pathways of dietary flavonoids and cinnamates. *Biochemical Society Transactions*. 28(2): 16-22.
- Wolfe, K.L. and R.H. Liu. 2007. Cellular antioxidant activity (CAA) assay for assessing antioxidants, foods, and dietary supplements. *Journal of Agricultural and Food Chemistry*. 55(22): 8896-8907.
- Wolfe, K., X. Wu and R.H. Liu. 2003. Antioxidant activity of apple peels. *Journal of Agricultural and Food Chemistry*. 51: 609-614.

- Wolfe, K.L., X. Kang, X. He, M. Dong, Q. Zhang and R.H. Liu. 2008. Cellular antioxidant activity of common fruits. *Journal of Agricultural and Food Chemistry*. 56(18): 8418-8426.
- Wolffram, S., M. Block and P. Ader. 2002. Quercetin-3-glucoside is transported by the glucose carrier SGLT1 across the brush border membrane of the rat small intestine. *Journal of Nutrition*. 132: 630-635.
- Wolffram, S. 2002. Letter to the Editor: Reply to Arts, Sesink and Hollman. *Journal of Nutrition*. 132: 2824.
- World Cancer Research Fund and American Institute for Cancer Research. 2007. Food, Nutrition, Physical Activity, and the Prevention of Cancer: A Global Perspective. AIRC, Washington, DC.
- Wu, A.H., M.C. Pike and D.O. Stram. 1999. Meta-analysis: dietary fat intake, serum estrogen levels, and the risk of breast cancer. *Journal of the National Cancer Institute*. 91: 529-534.
- Wu, Y., K. Ciu, K. Miyoshi, L. Henninghausen, J.E. Green, J. Setser, *et al.* 2003. Reduced circulating insulin-like growth factor I levels delay the onset of chemically and genetically induced mammary tumors. *Cancer Research*. 63(15): 4384-4388.
- Wynder, E.L. and E.A. Graham. 1950. Tobacco smoking as a possible etiologic factor in bronchogenic carcinoma. *Journal of the American Medical Association*. 143: 329-336.
- Xia, Y., L. Jin, B. Zhang, H. Xue, Q. Li and Y. Xu. 2007. The potentiation of curcumin on insulin-like growth factor-1 action in MCF-7 human breast carcinoma cells. *Life Sciences*. 80(23): 2161-2169.
- Yakar, S., J.L. Liu, B. Stannard, *et al.* 1999. Normal growth and development in the absence of hepatic insulin-like growth factor I. *Proceedings of the National Academy of Sciences*. 96: 7324-7329.
- Yamamoto, Y. and R.B. Gaynor. 2004. IkappaB kinases: key regulators of the NF-kappaB pathway. *Trends in Biochemical Sciences*. 29: 72-79.

- Yang, X.F., W.G. Beamer, H. Huynh and M. Pollak. 1996. Reduced growth of human breast cancer xenografts in hosts homozygous for the lit mutation. *Cancer Research*. 56(7): 1509-1511.
- Yoon, H. and R.H. Liu. 2008. Effect of 2alpha-hydroxyursolic acid on NF-kappa B activation induced by TNF-alpha in human breast cancer MCF-7 cells. *Journal of Agricultural and Food Chemistry*. 56(18): 8412-8417.
- Yoon, H. and R.H. Liu. 2007. Effect of selected phytochemicals and apple extracts on NF-kB activation in human breast cancer MCF-7 cells. *Journal of Agricultural and Food Chemistry*. 55(8): 3167-3173.
- Youngren, J.F., K. Gable, C. Penaranda, B.A. Maddux, M. Zavodovskaya, M. Lobo and M. Campbell. 2005. Nordihydroguaiaretic acid (NDGA) inhibits the IGF-I and c-erbB2/HER2/neu receptors and suppresses growth in breast cancer cells. *Breast Cancer Research and Treatment*. 94: 37-46.
- Zhang, S. and M.E. Morris. 2003. Effects of the flavonoids biochanin A, morin, phloretin, and silymarin on P-glycoprotein-mediated transport. *Journal of Pharmacology and Experimental Therapeutics*. 304(3): 1258-1267.
- Zhang, S., D.J. Hunter, M.R. Forman, B.A. Rosner, F.E. Speizer, G.A. Colditz and J.E. Manson. 1999. Dietary carotenoids and vitamins A, C, and E and risk of breast cancer. *Journal of the National Cancer Institute*. 91(6): 547-556.
- Zhao, M., B. Yang, J. Wang, Y. Liu, L. Yu and Y. Jiang. 2007. Immunomodulatory and anticancer activities of flavonoids extracted from litchi (*Litchi chinensis* Sonn.) pericarp. *International Immunopharmacology*. 7(2): 162-166.
- Zhou, J., T.R. Kumar, M.M. Matzuk and C. Bondy. 1997. Insulin-like growth factor I regulates gonadotropin responsiveness in the murine ovary. *Molecular Endocrinology*. 11: 1924-1933.
- Zhou, J-R., L. Yu, Z. Mai and G.L. Blackburn. 2004. Combined inhibition of estrogen-dependent human breast carcinoma by soy and tea bioactive components in mice. *International Journal of Cancer*. 108(1): 8-14.

## CHAPTER 2

### A MODIFIED METHYLENE BLUE ASSAY FOR ACCURATE CELL COUNTING

#### **ABSTRACT**

Cell counting is a common technique in cellular and molecular biology research applications, such as cell culture maintenance, cell plating, cell growth and cell doubling time determinations, as well as cell proliferation and cytotoxicity measurements. Many commonly employed cell counting methods exhibit limitations that influence resulting accuracy or versatility. For example, the trypan blue method typically underestimates cell numbers in culture, and the Lowry protein assay can be influenced by cell cycle. An urgent need exists for a method of cell counting that is both accurate and versatile. This work intended to explore an adaptation of the methylene blue assay to overcome the existing limitations of the procedure, enabling application to a broader range of cell densities and various cell culture plates. This new methylene blue assay was found to be more efficient, accurate and sensitive. A linear relationship ( $r^2 > 0.99$ ) was established between cell number and absorbance at a 570 nm wavelength when the new methylene blue assay was applied to three cell lines (HepG2, Caco-2, and MCF-7) plated in a broad range of cell densities ( $0.5 \times 10^4$  to  $2.5 \times 10^6$ ) in 4 different types of culture plates (6-, 12-, 24-, and 96-well plates). These results demonstrated that the linear relationship between methylene blue absorbance and cell number can be maintained at high cell densities. Growth curves were determined using both the trypan blue and methylene blue methods. At each time point in the HepG2 growth curve, the cell count obtained using the trypan blue assay was statistically significantly lower than that obtained using the methylene blue assay ( $p < 0.05$ ). The same was true for the Caco-2 growth curve at all time points ( $p < 0.05$ ).

except the 0 hour. The methylene blue method proposed in this paper may serve as a direct, automated counting method for cells grown in any type of culture plate. The methylene blue assay has clear advantages over traditional methods for several applications, including most assays that require an accurate cell count, such as bioavailability and cytotoxicity assays, and more basic experiments such as growth curve or doubling time determination for a particular cell line or in response to a treatment. The methylene blue assay modified here is a powerful tool for any application requiring a versatile, efficient, and accurate method of cell counting, especially in the research of natural products, bioactive compounds, phytochemicals, functional foods and nutraceuticals.

## **INTRODUCTION**

Cell counting is a necessary technique in cellular and molecular biology research, such as cell culture maintenance, cell plating, cell growth and cell doubling time determinations, and cell proliferation and cytotoxicity measurements (Sun and Liu, 2006; Wolfe and Liu, 2007; Yoon and Liu, 2007). Accurate cell counts are essential in both quantitative and qualitative experiments and interpretation of results depends on accurate initial and final cell counts. Consistent cell counting throughout an experiment minimizes variation.

Currently, the most commonly used method of direct cell counting is trypan blue staining followed by microscopic quantification using a hemacytometer. This technique involves trypsinizing adherent cells, removing cells from culture, centrifugation and re-suspension, staining with trypan blue, and cell counting. Some cells may not survive the trypsinization step due to degradation of chromatin by trypsin. The loss of any number of cells is a source of underestimated cell numbers, making a satisfactory cell count difficult to attain. The reliability of the trypan blue

method depends on a uniform, single cell suspension free of clumps, which would certainly interfere with the accuracy of the counting calculations. Cell counting on a hemacytometer can be very subjective, with results varying from one researcher to the next. There is an urgent need for an objective cell-counting method that will produce accurate and consistent cell numbers. An assay which measures cells directly on the plate in which they were grown and treated would eliminate these shortcomings.

An alternative method that is more objective than the trypan blue cell counting method is the Lowry Protein assay (Lowry *et al.*, 1951). In this assay, protein content indirectly measures cell numbers. Such a method relies on the generalization that cell number and cellular protein are directly proportional. Generally speaking, the more cells present, the more protein will be detected. However, total protein content in a cell fluctuates over time. For example, cell cycle proteins are synthesized and degraded regularly as the cell progresses through the cell cycle (Evans *et al.*, 1983; Glotzer *et al.*, 1991; King *et al.*, 1996). External conditions can also influence production of proteins in a cell. Protein production decreases dramatically while serum-starved cells revert to G0 (Liu *et al.*, 1994; Shirane *et al.*, 1999; Liu *et al.*, 2004; Sun and Liu, 2006). Therefore, the same number of cells can produce varying levels of protein depending on the distribution of cell numbers in each stage of the cell cycle. In addition, the protein of collagen-treated plates can interfere with protein-detection assays. In such situations protein content in cell culture cannot be directly correlated to cell numbers.

Lagneau, *et al.* (1977) introduced an alternative method, the methylene blue assay, to measure cytotoxicity. The study measured the cytotoxicity of BCG-treated rat macrophages on intestinal cancer cells *in vitro*. The cells to be measured were stained with methylene blue. The stain was eluted from the cells and measured spectrophotometrically, yielding a quantitative measure of cytotoxicity.

Finlay *et al.* (1984) introduced a modified methylene blue staining technique for measuring cytotoxicity of growth inhibiting drugs on adherent cell lines using a spectrophotometric micro-plate reader. Two human colon adenocarcinoma cell lines, HCT-8 and HT-29, were plated at a density of  $1 \times 10^3$  cells/well in 96-well plates. Two days later, cells were treated with varying concentrations of the growth inhibiting drugs doxorubicin, amsacrine derivatives, and nitracrine. After four days the cells were stained with methylene blue (5 g/L of 50% (v/v) ethanol:water, 0.1 mL/well) for 30 minutes, rinsed, and solubilized overnight with 1% Sarkosyl in PBS. Absorbances were read on an ELISA microplate reader at a wavelength of 620 nm. The absorbance reading is a reflection of the number of surviving cells, which remain attached to the plate as cells killed due to the cytotoxicity will detach from the plate. Finlay *et al.* (1984) reported that absorbance correlates with cell numbers and that this methylene blue assay is more efficient than the time-consuming processes of trypan blue counting or preparing samples for an electronic particle counter. The authors note that while some drugs may cause an increase in average cell size as cells accumulate in G2 phase, this increase does not cause a significant difference in results between the methylene blue assay and direct cell counting. Applications of this technique include cytotoxicity assays for various chemicals on adherent cell lines, as well as interactions between these cytotoxic agents and other compounds such as hormones, nutrients, and DNA repair inhibitors (Finlay *et al.*, 1984). While Finlay *et al.* demonstrated the correlation between methylene blue absorbance and cell number only for cell numbers suited for 96-well plates ( $1 \times 10^3$  cells/well), the use of 96-well plates and small cell numbers make this assay well-suited for preliminary cytotoxicity screenings.

Oliver *et al.* (1989) reported a modification of Lagneau's original methylene blue assay to be used with microwell plates. Cells plated in 96-well plates were first fixed with 10% formol saline for 30 minutes. After 30 minutes of staining with 1%

(w/v) methylene blue in 0.01 M borate buffer (pH 8.5), wells were rinsed in 0.01 M borate buffer and eluted with 1:1 (v/v) ethanol and 0.1 M HCl. Absorbances were read at 650 nm on an automated microplate photometer. Oliver *et al.* found that absorbances correlated with cell numbers in the three cell lines assayed (Rat-1, fetal rat lung fibroblast; IMR90, human fetal lung; and HFL, human fetal lung fibroblast). The authors used this methylene blue assay to observe the influence of growth factors on cell proliferation (e.g. PDGF, platelet-derived growth factor), and proposed to use the assay for observing *in vitro* effects of cell growth stimulators or inhibitors. The adaptation of a methylene blue assay for use with an automated plate reader has contributed a great deal of ease and efficiency to current cytotoxicity and cell proliferation assays. The principle drawback, however, is that the assay is limited to cell densities ranging from  $2 \times 10^3$  to  $4 \times 10^4$  cells per well, depending on the cell line (Oliver *et al.*, 1989).

The proposed cell counting methods discussed above each exhibit limitations that influence resulting accuracy or versatility. The trypan blue method underestimates cell numbers in culture, the Lowry protein assay can be influenced by cell cycle, and current automated methylene blue assays are limited to cultures in 96-well plates. An urgent need exists for an accurate and versatile method of cell counting. Our objective is to explore an adaptation of the methylene blue assay to overcome the existing limitations of the procedure and to enable application to a broader range of cell densities and larger cell culture plates. Currently there is no report of such an assay that can be applied to 6-, 12-, or 24-well cell culture plates. The methylene blue method proposed in this paper may serve as a direct, automated counting method for cells grown in any type of culture plate.



## MATERIALS AND METHODS

**Chemicals.** Methylene blue was purchased from BioQuest (Bedford, MA), glutaraldehyde from Sigma Chemical Co. (St. Louis, MO), ethanol from Mallinckrodt (Phillipsburg, NJ), and acetic acid from Fisher Scientific (Pittsburgh, PA). Williams' Medium E (WME), Dulbecco's Modified Eagle Medium (DMEM),  $\alpha$ -Minimal Essential Medium, fetal bovine serum (FBS), Hank's Balanced Salt Solution (HBSS) and Dulbecco's Phosphate Buffered Saline were purchased from Gibco Life Technologies (Grand Island, NY).

**Cell lines.** The HepG2 human liver cancer cells, Caco-2 human colon cancer cells, and MCF-7 human breast cancer cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD).

**Cell Culture.** All cell culture media contained 5% FBS, 10 mM Hepes, 50 units/mL penicillin, 50  $\mu$ g/mL streptomycin, and 100  $\mu$ g/mL gentamicin. HepG2 cells were maintained in WME containing an additional 5  $\mu$ g/mL insulin, 2  $\mu$ g/mL glucagon and 0.05  $\mu$ g/mL hydrocortisone. Caco-2 cells were maintained in DMEM, and MCF-7 cells were grown in  $\alpha$ -MEM containing 10% FBS. Cells were maintained at 37°C, in a 5% CO<sub>2</sub> atmosphere, and passaged before reaching confluence (Liu and Sun, 2003).

**Color Formation Stability.** HepG2 cells were plated in 12-well plates at a density of  $7.5 \times 10^5$  cells per well. After 4 hours of incubation at 37°C in 5% CO<sub>2</sub>, the media was aspirated and each well was gently rinsed with PBS. Cells were stained and fixed by adding 300  $\mu$ L methylene blue solution (HBSS + 1.25% glutaraldehyde + 0.6% methylene blue) to each well. After 1 hour of incubation at 37°C in 5% CO<sub>2</sub>, wells were rinsed with distilled water. Plates were drained and briefly air-dried before addition of 600  $\mu$ L elution solution (50% ethanol + 49% PBS + 1% acetic acid) to each well for 15 minutes at room temperature. After elution was complete, solutions

from each well were transferred to individual microcentrifuge tubes and centrifuged for 3 minutes at 12,000 g. The solutions were then transferred in duplicate to a 96-well plate (200  $\mu$ L/well) and read on an ELISA plate reader (Dynex Technologies, Chantilly, VA) at a wavelength of 570 nm. Samples were read at 0, 6, 12 and 20 minutes after centrifugation in order to determine the stability of color formation.

**Methylene Blue (MB) Staining.** HepG2 cells were plated in 12-well plates at a density of  $7.5 \times 10^5$  cells per well, three wells per treatment. Cells were fixed and stained with methylene blue for durations of 0, 5, 15, 30, 45 and 60 minutes at a constant volume of 300  $\mu$ L/well. After staining and rinsing, each sample was placed at 4°C until all subsequent samples were collected. Before adding the elution solution, all samples were rinsed once more, drained and air-dried, in order to minimize moisture variation among the fixed samples. To each well 600  $\mu$ L of elution solution were added, and plates were placed on a shaker at room temperature for 60 minutes. Elution solutions were then centrifuged in microcentrifuge tubes for 3 minutes at 12,000 g, and transferred in duplicate to a 96-well plate, 200  $\mu$ L/well. The 96-well plate was read on the plate reader at a wavelength of 570 nm.

**Elution Kinetics.** HepG2 cells were plated in a 12-well plate, at  $7.5 \times 10^5$  cells in 0.75 mL media per well. Cells were left for 3 hours to attach, then stained and fixed with methylene blue for 60 minutes (300  $\mu$ L/well), rinsed 6 times with distilled water, and eluted with 600  $\mu$ L/well of elution solution for varying durations (0, 5, 15, 30, 45 and 60 minutes). As the elution solutions were collected from the samples at each time point, the solutions were placed in microcentrifuge tubes. After the final sample was collected, all samples were micro-centrifuged at 12,000 g for 3 minutes. Samples were then transferred to a 96-well plate, 200  $\mu$ L/well, and 2 wells/sample. The plate was read on the plate reader at a wavelength of 570 nm.

**Modified Methylene Blue Assay for Cell Counting.** Based on the previously described experiments, we have designed a modified methylene blue assay for a broad range of cell counting. This method can be directly applied to adherent cells growing in any type of culture plate. The modified protocol was as follows: Growth media was removed and each well rinsed with PBS. The appropriate volume of methylene blue solution (HBSS + 1.25% glutaraldehyde + 0.6% methylene blue) was added to each well of the culture plate, according to Table 2.1. After incubation at 37°C for 60 minutes, the methylene blue solution was removed from the wells, then plates were rinsed by gentle submersion in distilled water 6 times. The excess water was allowed to drain and the plate was briefly air-dried. The appropriate volume of elution solution (50% ethanol + 49% PBS + 1% acetic acid) was added to each well (Table 2.1). After 15 minutes on a plate rotator at room temperature, the elution solutions were centrifuged at 12,000g for 3 minutes. The solutions were transferred to a 96-well plate: 2 wells per sample, 200  $\mu$ L into each well. Plates were read using a micro-plate reader at 570 nm wavelength. A standard curve was established by plating a range of cell densities of particular cell line in the desired culture plate. The equation of the line was used to convert absorbance of each sample to cell number.

**Table 2.1. Recommended cell numbers and reagent volumes per well for methylene blue assay.**

Cell Culture Plate	Cell Number Plated	Methylene Blue ( $\mu$ L)	Elution Solution ( $\mu$ L)
96-well	0.5 to $5 \times 10^5$	50	100
24-well	0.5 to $3 \times 10^5$	200	400
12-well	1 to $10 \times 10^5$	300	600
6-well	0.65 to $2.5 \times 10^6$	1000	1000

**Broad Range Standard Curves.** Three cell lines, Caco-2, HepG2 and MCF-7, were used to demonstrate the application of this modified assay to broad ranges of cell

densities ( $5 \times 10^3$  to  $2.5 \times 10^6$ ) in various cell culture plates (6-, 12-, 24- and 96-well plates). In each of the four types of cell culture plates, the three cells lines were plated at various densities within the ranges described in Table 2.1. Cells were plated, allowed to attach (4 hours for HepG2; 6 hours for Caco-2 and MCF-7), then analyzed by the MB assay. Sample solutions were read spectrophotometrically on a plate reader at a wavelength of 570 nm. The absorbance of each well was plotted versus initial cell density, demonstrating the linear relationship between absorbance at 570 nm and cell number. In the 6-well plates, the absorbance of these samples was out of range of the plate reader. In these cases, each sample was diluted by an addition of 100  $\mu$ L of fresh elution solution to 100  $\mu$ L sample solution in each well of the 96-well plate. The absorbance of each sample was then corrected for this 1:1 dilution.

**Trypan Blue (TB) Cell Counting.** In order to evaluate the accuracy of trypan blue counting, actual counts were plotted versus the predicted cell number. A set of Caco-2 cells identical to that of the broad range standard curve described above was plated. The cells were left to attach for 6 h (same duration as for methylene blue assay), harvested by trypsinization, and 50  $\mu$ L of cell solution were mixed with 450  $\mu$ L of 0.4% trypan blue solution prepared in 0.85% saline. The cells in 10  $\mu$ L of this suspension were directly counted on a hemacytometer. The average cell count of 4 fields represents the number of cells per mL of cell solution, and was used to determine the total number of cells from each well.

**Comparison of Cell Growth Curves Determined by Methylene Blue and Trypan Blue Counting.** On the first day of this 5-day experiment, a standard curve of absorbance versus cell number was established. Briefly, Caco-2 (or HepG2) cells were plated at densities within the ranges found in Table 2.1. These cell densities were plotted against the corresponding absorbance results of the methylene blue assay. The equation of the line of the standard curve was then used to convert absorbance of

samples to cell number. Cells for the growth curve were plated at a fixed density of  $7.5 \times 10^5$  per well in 12-well plates. Every 24 hours for 5 days, 3 wells were subjected to the methylene blue assay, and absorbance was converted to cell number using the equation of the line of the standard curve ( $r^2=0.9918$ ). An additional 3 wells were counted using trypan blue counting. Cell counts determined by conversion of absorbance to cell number via the equation of the line of the standard curve were compared to cell counts determined by direct trypan blue counting.

**Applications in Phytochemical Research: Antiproliferative Activity.** We used this modified methylene blue assay to test the anti-proliferative activity of whole apple extract. Our lab has previously described the method of extracting soluble free phenolics from whole foods (Eberhardt *et al.*, 2000; Dewanto *et al.*, 2002) and has used the MTT assay to demonstrate anti-proliferative activity of various fruit and vegetable extracts (Chu *et al.*, 2002; Sun *et al.*, 2002; Sun and Liu, 2006). *Standard Curve.* A standard curve of MCF-7 cells was plated under cell culture and media conditions as described earlier. Cells were plated in a 96-well plate at densities (in triplicate wells) ranging from 0 to  $8 \times 10^4$  cells per well. Cells were incubated at 37°C for 6 hours, at which point the cells were counted by the modified methylene blue method. *Apple Extract: Anti-Proliferation Dose Response.* MCF-7 cells were plated under the same conditions, at a cell density of  $2.5 \times 10^4$  cells per well. Six hours after plating, cells were treated with varying doses of apple extract in  $\alpha$ -MEM with 10% FBS. This medium alone with no apple extract served as the control, since no extraction solvent remained in the apple extract. Forty-eight hours after treatment, the medium of the experimental cells was replaced with fresh medium containing the appropriate dose of apple extract. Cells were stained and assayed by the methylene blue method 72 hours after the initial treatment.

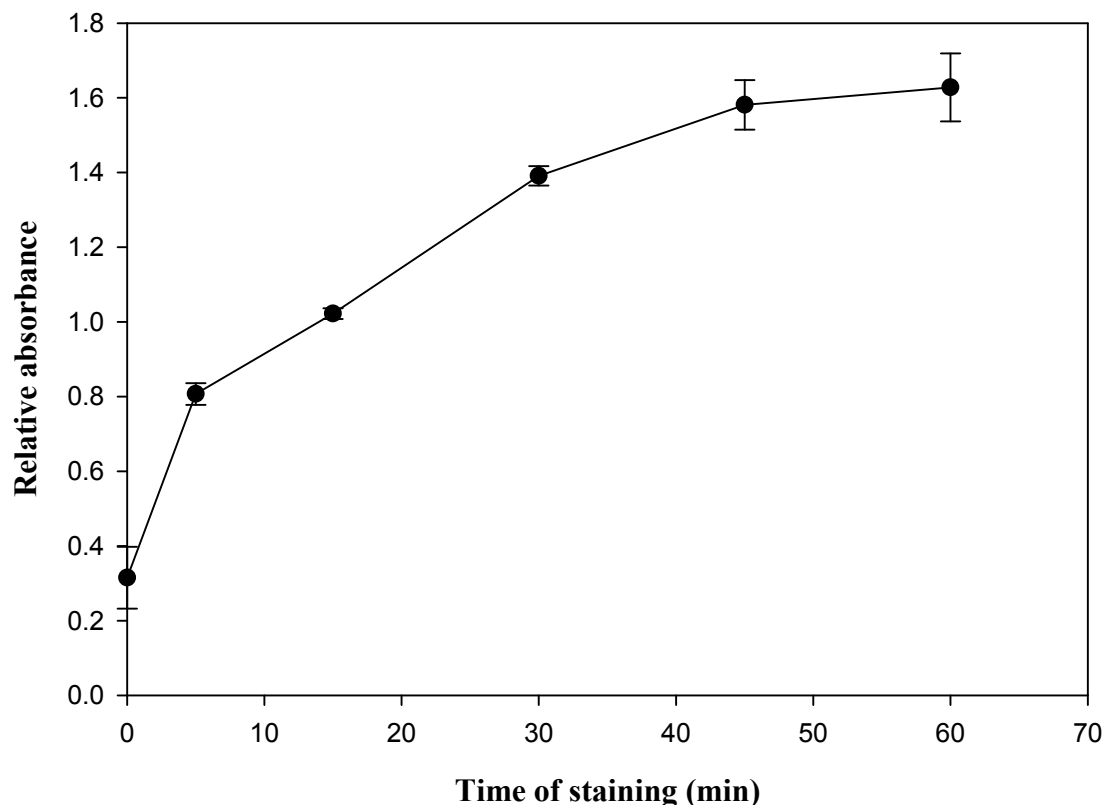
**Statistics.** Statistical analysis was done using Minitab Release 12 software (Minitab Inc., State College, PA). Values were compared using a paired-t test, with a significance level of  $p < 0.05$ .

## RESULTS

**Optimization of Method.** *Color Formation Stability.* Variation between absorbance readings at 0, 6, 12 and 20 minutes was virtually eliminated (data not shown) when the elution solution centrifugation step was added. Absorbance remains stable for at least a 30-minute period.

*Methylene Blue Staining Kinetics.* The methylene blue stain was introduced to the cells and immediately removed for the 0 minute staining data point. The absorbance value of the methylene blue eluted from these 0 minute cells was 0.315, or 19.3% of that measured at 60 minutes staining duration (absorbance of 1.628) (Figure 2.1). After 5 minutes staining duration the absorbance reached 49.6% that of the 60 minute time point. The measured absorbance had reached 62.8% by 15 minutes and 85.4 % by 30 minutes. This curve began to plateau after 45 minutes as staining began to reach saturation (97.1%), indicating that 60 minutes was indeed the optimal staining duration.

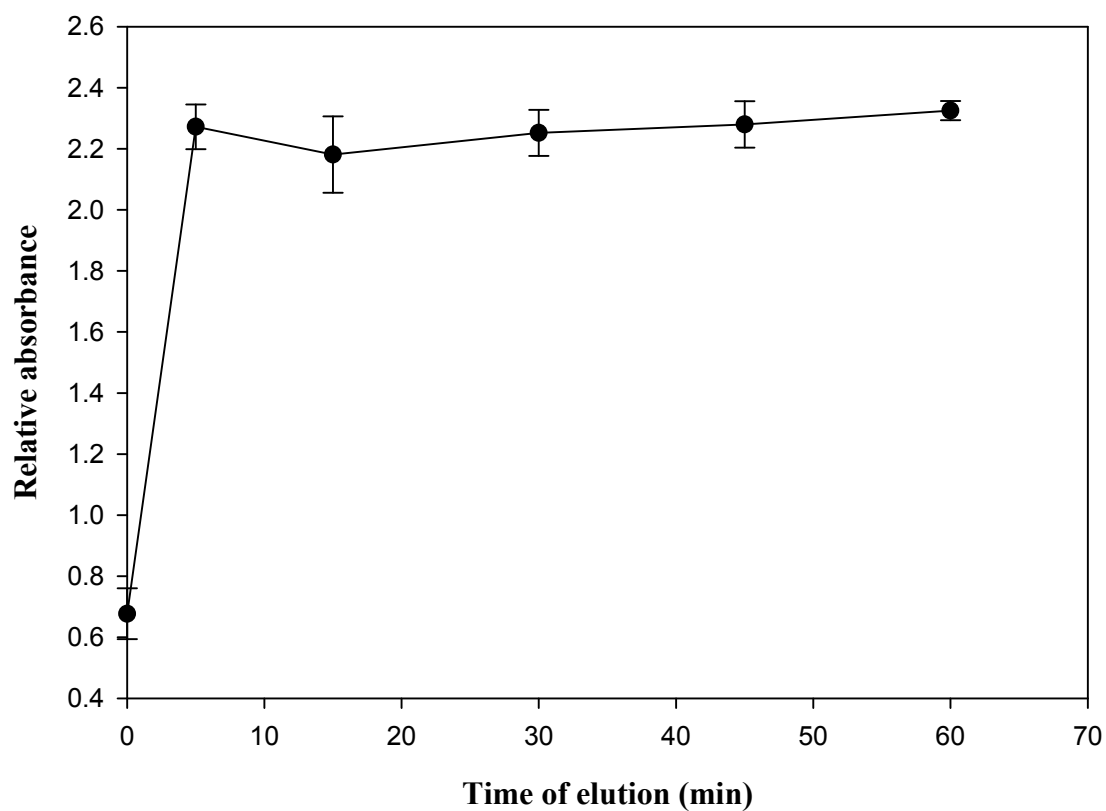
*Elution Kinetics.* Absorbance readings were taken after various durations of elution of the methylene blue solution from the cells. After 0 minutes of elution, (removing the elution solution immediately after placing it in the well) the mean absorbance was 0.677. Elution durations of 5, 15, 30, 45 and 60 minutes yielded relatively constant absorbance readings ranging between 2.181 and 2.325 (Figure 2.2). From these results, it was determined that 15 minutes was a sufficient elution duration.



**Figure 2.1. Methylene Blue Staining Kinetics.** The duration of methylene blue staining was varied from 0 to 60 minutes. Variation over time among readings of the same samples was minimized by centrifugation of samples prior to reading. The curve began to plateau after 45 minutes, indicating the point at which staining began to reach saturation (mean  $\pm$  SD,  $n = 3$ ).

**Broad Range Standard Curves.** Cells were plated in 6-well plates (Figure 2.3a) at densities between  $0.75$  and  $2.5 \times 10^6$  cells per well. The methylene blue absorbance of all three cell lines increased linearly with cell numbers producing  $r^2$  values of  $0.9978$  for HepG2,  $0.9922$  for Caco-2, and  $0.9894$  for MCF-7. Cell plated in 12-well plates (Figure 2.3b), in densities ranging from  $1$  to  $10 \times 10^5$  per well, demonstrated the same linear correlation between absorbance and cell number as exhibited in 6-well plates. The standard curves in these 12-well plates yielded  $r^2$  values of  $0.9986$ ,  $0.9918$ , and  $0.9987$  for HepG2, Caco-2, and MCF-7 cells, respectively. Cell densities in 24-well plates (Figure 2.3c) ranged from  $0.5$  to  $3 \times 10^5$

cells per well and produced standard curves with  $r^2$  values of 0.9874, 0.9858, and 0.9999 for HepG2, Caco-2, and MCF-7 cells, respectively. The 96-well plates (Figure 2.3d) contained cells in densities between  $0.5$  and  $5 \times 10^4$  per well and produced standard curves with the  $r^2$  values of 0.9938, 0.9914, 0.9940 for HepG2, Caco-2, and MCF-7 cells, respectively. The HepG2 cells had the highest methylene blue absorbance followed by MCF-7 cells, with Caco-2 cells having the lowest, in all cases except the 6-well plates, where the MCF-7 cells had the highest followed by HepG2 cells.



**Figure 2.2. Methylene Blue Elution Kinetics.** HepG2 cells were fixed and stained according to the modified methylene blue assay. Elution times were varied from 0 to 60 minutes (mean  $\pm$  SD,  $n = 3$ ).



**Figure 2.3.** Relationship of cell number to relative absorbance in three cell lines (HepG2, Caco-2, and MCF-7) and four different culture plates: 6-well (a), 12-well (b), 24-well (c) and 96-well (d) (mean  $\pm$  SD, n = 3).

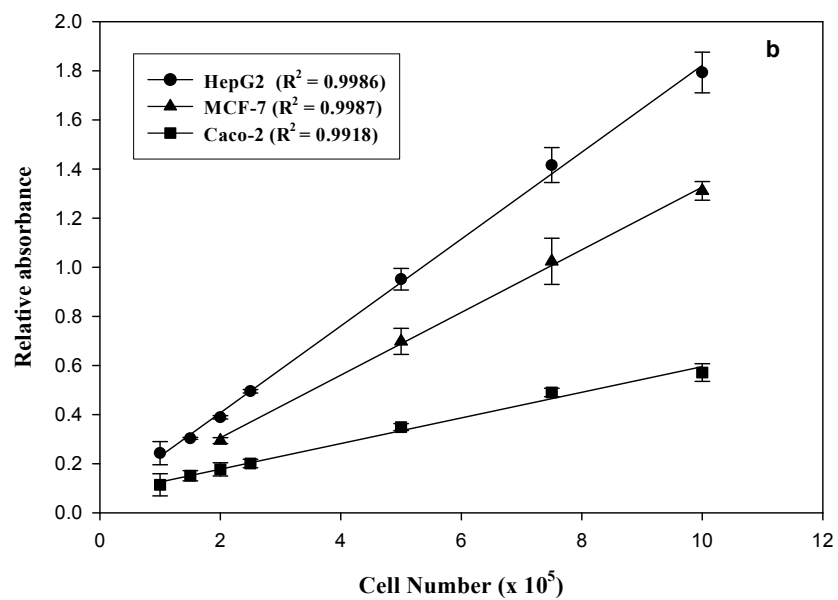
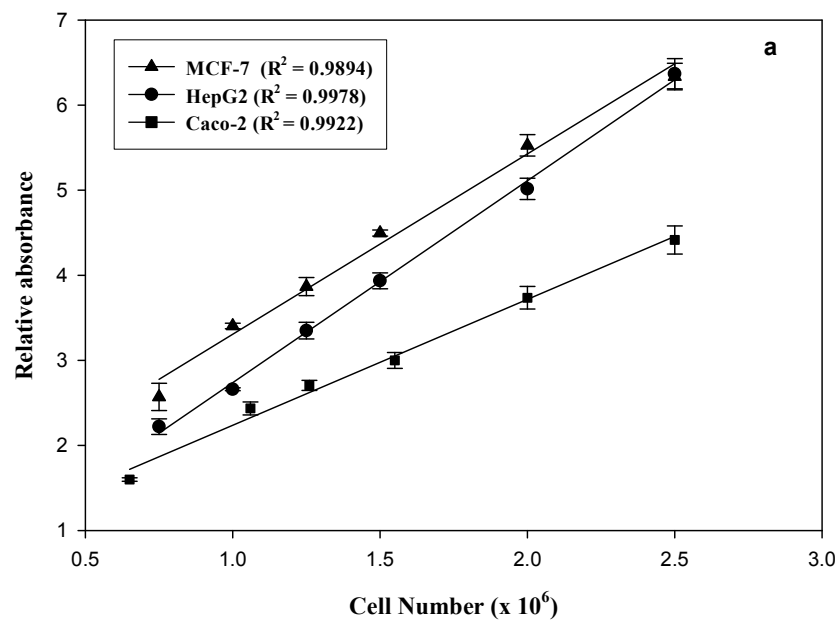
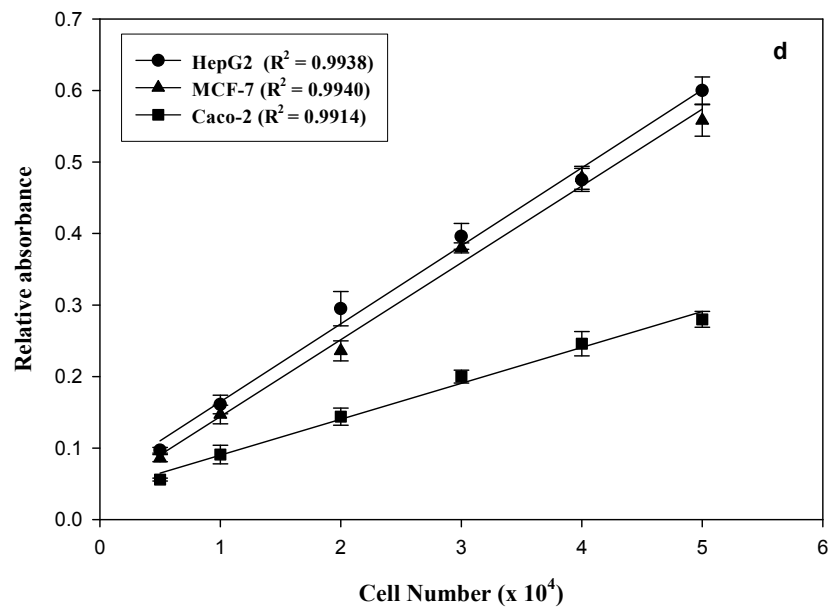
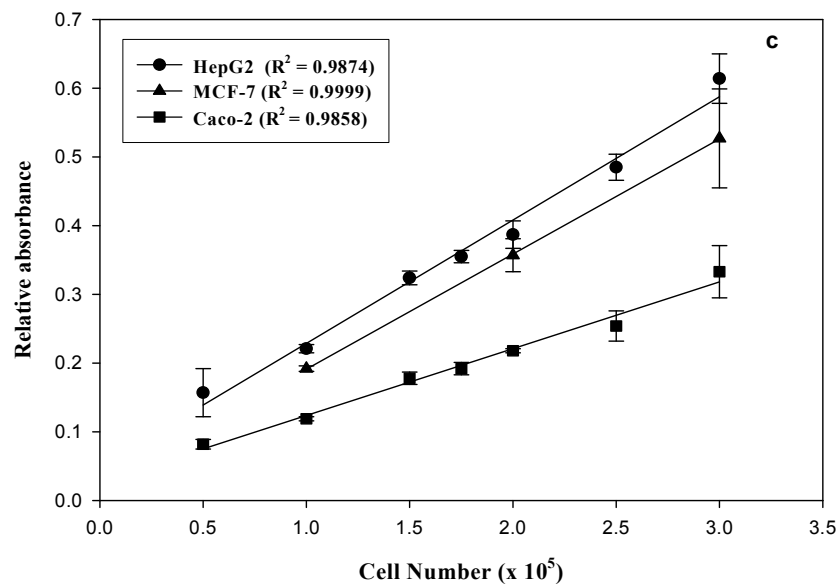
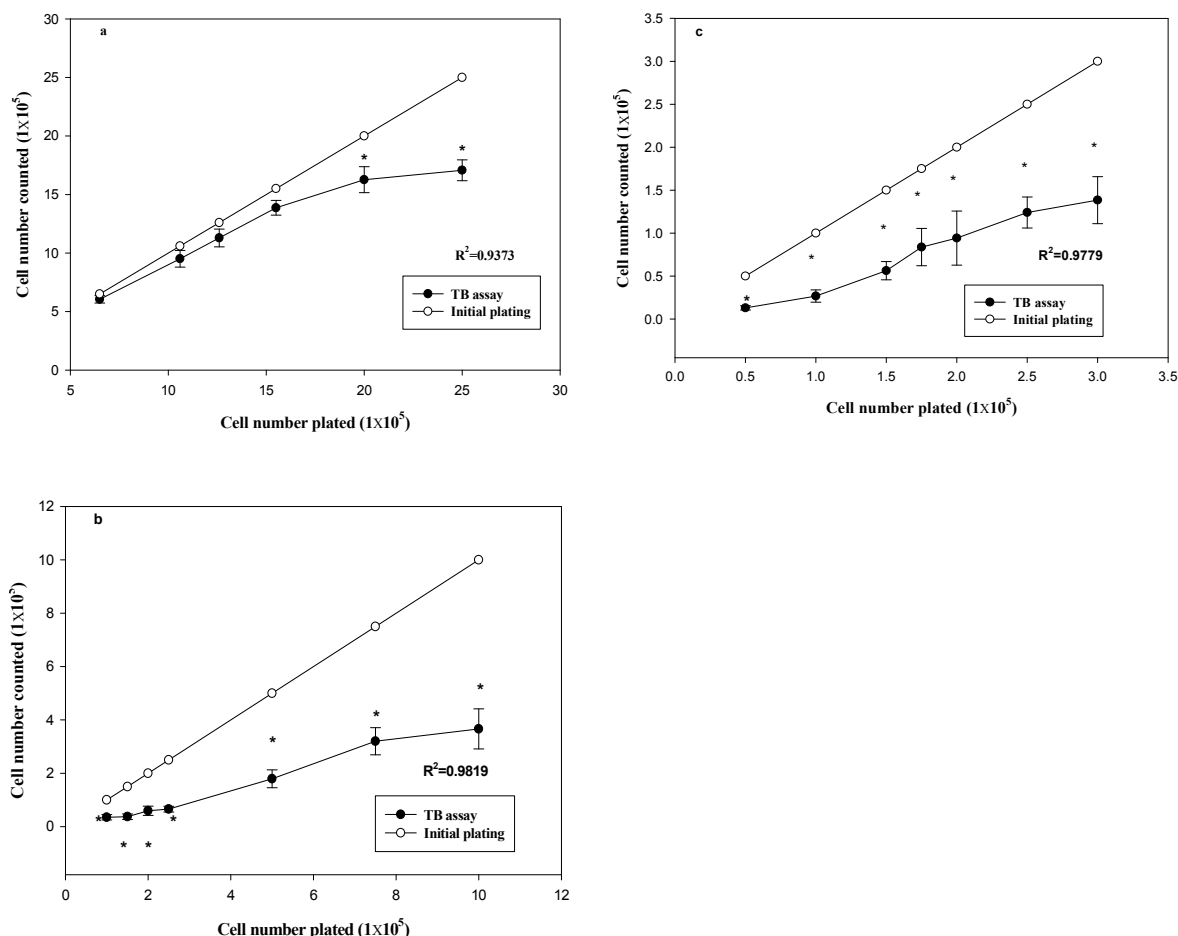


Figure 2.3 (Continued)





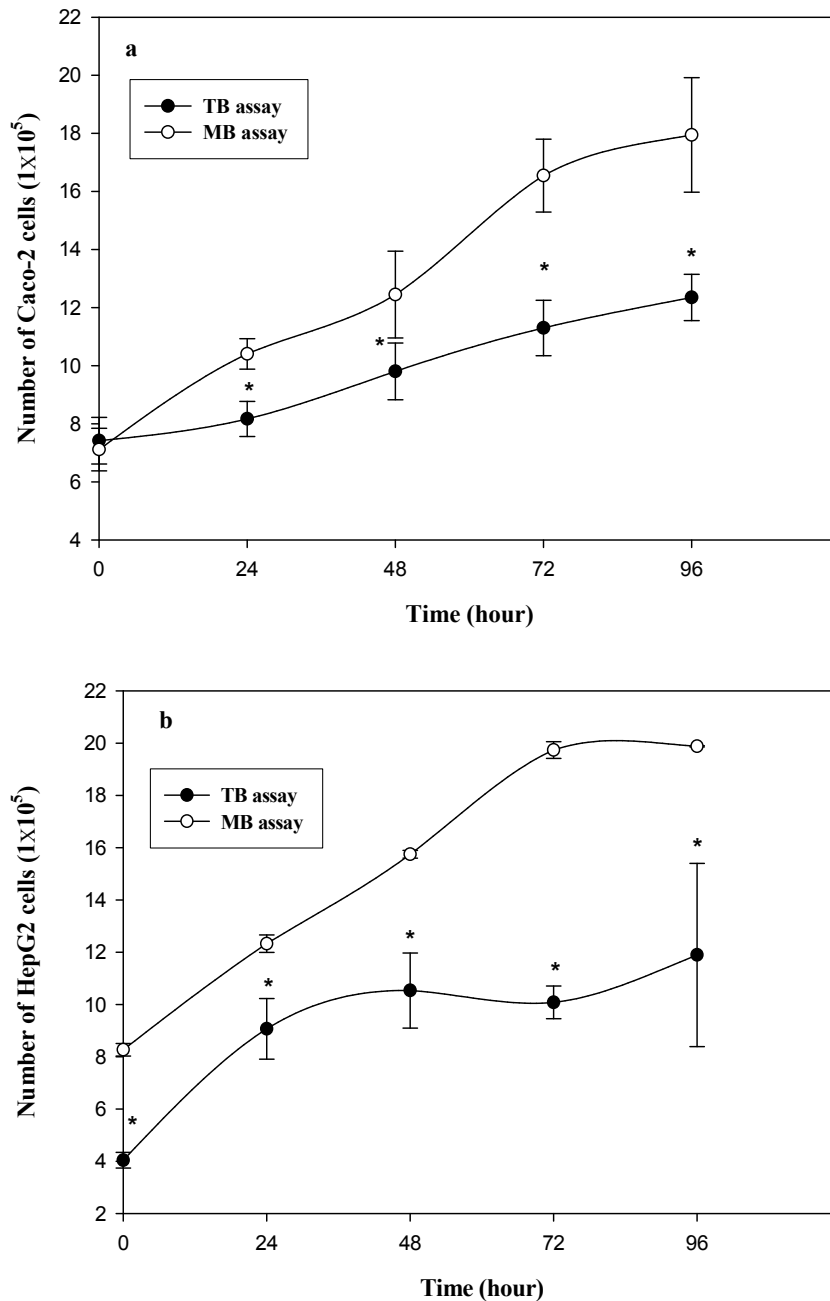
**Figure 2.4. Number of Caco-2 cells determined by the trypan blue cell counting assay (TB assay) compared to the number of cells initially plated.** (a) 6-well culture plate. (b) 12-well plate. (c) 24-well plate. An asterisk (\*) indicates a statistically significant difference ( $p < 0.05$ ) between trypan blue counting results (mean  $\pm$  SD,  $n = 3$ ) and the corresponding initial plating number.

**Trypan Blue Cell Counting.** The trypan blue (TB) counts for 6-well plates underestimated numbers by 7 to 32%, presumably because the number of cells lost from a larger well represents a smaller percentage (Figure 2.4a). In the 6-well plates, only the 2 highest cell densities ( $2 \times 10^6$  and  $2.5 \times 10^6$ ) had TB counts that were statistically significantly lower than the initial plating density ( $p < 0.05$ ). The TB counts consistently underestimated the initial plating density by between 50 and 75% for 12-

and 24-well plates (Figure 2.4b and 2.4c). Trypan blue counts were statistically significantly lower than initial plating density ( $p < 0.05$ ) for all cell densities in both the 24-well and 12-well plates. The cell numbers for 96-well plates were below the lower range for TB counting, illustrating one case where the MB assay may be more sensitive.

**Comparison of Cell Growth Curves Determined by Methylene Blue and Trypan Blue Counting.** A Caco-2 standard curve (not shown) was established over a range of  $1 \times 10^5$  to  $10 \times 10^5$  cells/well in a 12-well plate. New cells were then plated in 12-well plates at an initial density of  $0.75 \times 10^5$  cells/well. The two growth curves are compared in Figure 2.5a. The trypan blue method resulted in significantly lower Caco-2 cell counts at every time point except the 0 hour ( $p < 0.05$ ). Beginning at 24 hours, the difference between the two counting methods began to increase over time. At 24, 48, 72, and 96 hours, the TB cell count was 21.5, 21.2, 31.7, and 31.2% lower than that of the methylene blue method, respectively.

HepG2 cells were plated identically to that of the Caco-2 cells. The linear HepG2 standard curve used to convert absorbance to cell number produced an  $r^2$  of 0.9925. The growth curve in Figure 2.5b is a comparison of cell counts obtained using the methylene blue assay with those obtained using the trypan blue assay. At each time point in the HepG2 growth curve, the cell count obtained using the trypan blue assay was statistically significantly lower than that obtained using the methylene blue assay ( $p < 0.05$ ). The initial trypan blue count at 0 hours was 51.1% lower than the methylene blue count. At 24, 48, 72 and 96 hours, the trypan blue cell count was 26.4, 33.1, 48.9, and 40.2% lower, respectively, than that obtained using the methylene blue method.



**Figure 2.5.** Comparison of cell growth curves determined by the trypan blue assay (TB) and the modified methylene blue assay (MB) in both human Caco-2 colon cancer cells (a) and HepG2 liver cancer cells (b) *in vitro*. An asterisk (\*) indicates a trypan blue cell count is statistically significantly different ( $p < 0.05$ ) from the corresponding methylene blue count for that time point (mean  $\pm$  SD,  $n=3$ ).

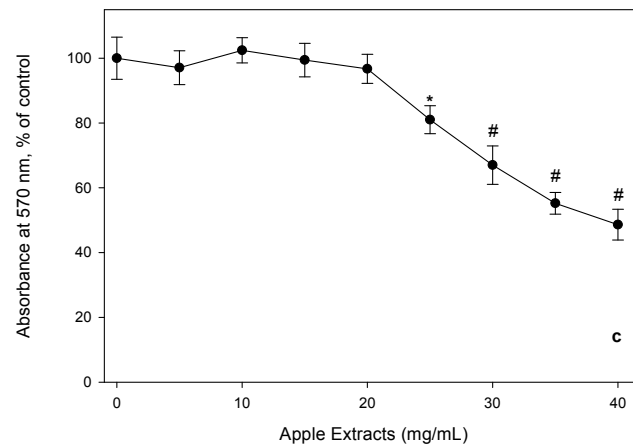
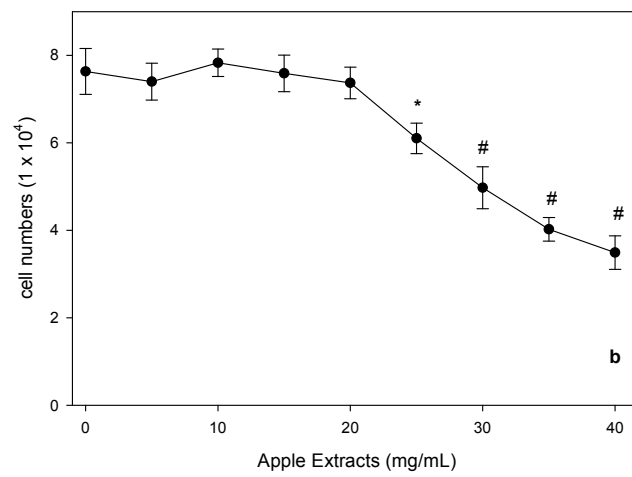
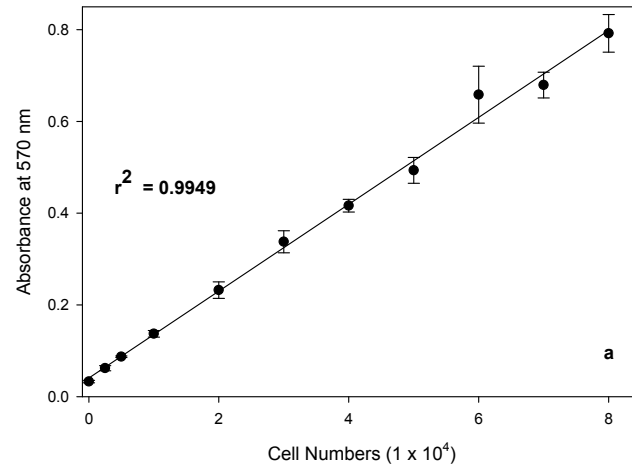
### **Applications in Phytochemical Research: Antiproliferative Activity.**

*Standard Curve.* Plotting the cell culture standard curve using the methylene blue assay shows absorbance at 570 nm is proportional to cell number plated with an  $r^2$  of 0.9949 (Figure 2.6a). This indicates that the absorbance measured by the methylene blue assay directly reflects cell number. The equation of this line can then be used to calculate cell numbers of experimental cells from the methylene blue absorbance. For example, 9 wells of cells plated at  $2.5 \times 10^4$  cells per well were assayed by methylene blue 6 hours after plating. Using the standard curve to calculate cell number based on absorbance generated a mean cell number of  $2.504 \times 10^4$  per well.

*Apple Extract: Anti-Proliferation Dose Response.* An application of the methylene blue direct cell counting assay is illustrated in Figure 2.6. Cell number of each treated group can be accurately calculated through the standard curve (Figure 2.6a) using absorbance. From Figure 2.6b one can see MCF-7 cell proliferation was significantly inhibited at 25 mg/mL ( $6.1 \times 10^4$ ) as compared to control ( $7.6 \times 10^4$ ,  $p < 0.05$ ). At a dose of 40 mg apple extract per mL, cell number was  $3.5 \times 10^4$ , ( $p < 0.01$ ). Alternatively, cell proliferation data can be presented as percent of control as illustrated in Figure 2.6c. This eliminates the need for the standard curve while producing the same results. Compared to control, treatment with apple extract significantly inhibits cell proliferation at doses of 25 mg/mL (81% of control,  $p < 0.05$ ) to 40 mg/mL (48.6% of control,  $p < 0.01$ ). Similar results are obtained whether the data is presented as cell number or percent of control, suggesting this methylene blue assay can be used either way.

**Figure 2.6. Applications in phytochemical research: Antiproliferative activity. (a) Cell Number Standard Curve.** MCF-7 cells were plated at varying densities, allowed to attach for 6 hours, then stained with methylene blue. The equation of the line ( $y = 0.0947x + 0.0407$ ,  $r^2 = 0.09949$ ) was used to convert the absorbance at 570 nm of experimental cells to cell number. **(b) Results Presented as Cell Number.** MCF-7 cells were plated in a 96-well plate at a density of  $2.5 \times 10^4$  cells per well, allowed to attach for 6 hours, then treated with varying doses of apple extract for 72 hours. Absorbance was determined by the modified methylene blue assay, then converted to cell number using the standard curve equation of the line (a). Apple extract significantly reduced cell number in a dose-dependent manner between 25 and 40 mg/mL (\*:  $p < 0.05$ ; #:  $p < 0.01$ ). **(c) Results Presented as Percent of Control.** Absorbance data from (b) was presented as a percent of the control, rather than converting to cell number. In this way, it is also clear that apple extract significantly reduced cell number in a dose-dependent manner between 25 and 40 mg/mL (\*:  $p < 0.05$ ; #:  $p < 0.01$ ).





## DISCUSSION

Various trials were carried out in order to optimize the methylene blue assay for accuracy, efficiency and convenience. Previously, absorbance readings of the elution solution would vary over time and eventually stabilize after about 20 minutes, indicating possible interference by suspended particles or proteins in the elution solution. An extra step of centrifuging the samples after elution virtually eliminated variation among the same readings over time.

The methylene blue staining kinetics curve (Figure 2.1) began to plateau after 45 minutes, indicating staining began to reach saturation around that point. The mean absorbance of samples appeared relatively constant between 5 and 60 minutes elution time (Figure 2.2). It is possible, then, that a shortened elution time, i.e. 15 or 30 minutes, would be no less effective than the 60 minutes prescribed by the current protocol. Our results demonstrated one hour was the optimal duration for methylene blue staining, while the elution step can be reduced to 15 minutes. Even with the added step of centrifugation, this modified assay is more time-efficient than the original procedure. The modified methylene blue assay is a quick and accurate cell counting method, especially useful when multiple samples must be counted simultaneously.

A linear relationship can be established between cell number and absorbance with an  $r^2 > 0.99$  (Figure 2.3). This new methylene blue assay was applied to three cell lines (HepG2, Caco-2, and MCF-7) plated in a broad range of cell densities ( $5 \times 10^4$  to  $2.5 \times 10^6$ ) in 4 different types of culture plates (Table 2.1). These results demonstrated that the linear relationship between methylene blue absorbance and cell number can be maintained at high cell densities. The significance of the modifications discussed in this paper is that an automated methylene blue assay can now be applied directly to cells grown and treated in any size culture plate.

Applications of this method include most assays which require an accurate cell count, such as bioavailability and cytotoxicity assays, and more basic experiments such as growth curve or doubling time determination for a particular cell line or in response to a treatment (Liu *et al.*, 2004; He and Liu, 2007; Yoon and Liu, 2007). One limitation of the method is that the cells stained with methylene blue are necessarily destroyed. The applications of the method, then, are limited to those in which determining a cell count is the end-point. Otherwise, an identical set of cells must be plated in parallel for the experiment.

The methylene blue assay has the advantage over the Lowry protein assay of being independent of the varying protein content of cells. Therefore, it is especially useful in situations where protein content cannot be used to reflect cell number, for example, experiments which run through the duration of the cell cycle (e.g. growth curves). Additionally, the method may be useful where conditions or treatments that may variably inhibit protein synthesis such as reverting cells to G0 (Sun and Liu, 2006). In these cases, protein content may vary and would not reflect the actual number of cells in the culture. Under certain conditions, then, it may not be possible to correlate protein content with cell number, especially across treatments which may affect protein levels. Other limitations of the protein assay include lack of differentiation between viable and dead cells, and interference of the protein on collagen-treated plates. The methylene blue assay offers the advantage of fixing and staining the cells in the very wells in which they were grown and treated. In the TB assay, cells are destroyed by trypsinization and lost by transfer steps, a distinct limitation that the methylene blue assay overcomes.

The MTT assay (Liu and Sun, 2003) and a subsequent variation, the MTS assay (Mossmann, 1983; Liu *et al.*, 2002), are two common cytotoxicity assays, both of which are based on the assumption that enzyme activity is indicative of cell

number. Enzyme levels in cells, however, fluctuate over time or under different conditions. For instance, mitochondrial dehydrogenase activity, measured by the MTT assay, is cell-type specific (Laville *et al.*, 2004).

The DNA thymidine assay can be used to convert the measure of thymidine incorporated by the cells into a cell number. The process of thymidine uptake, however, occurs only during DNA synthesis, the S phase of the cell cycle. This assay, therefore, is more suited to measuring DNA synthesis than actual cell numbers. Another limitation of this assay is the use of the radioactive isotope  $^3\text{H}$ . For reasons such as occupational exposure and waste disposal, a non-radioactive alternative would certainly be desirable (Yang *et al.*, 1996; Laville *et al.*, 2004).

A more recent method which claims to have several advantages over DNA  $^3\text{H}$  thymidine uptake or the MTS assay is an acid phosphatase assay, in which cytosolic acid phosphatase activity is used as a measure of cell proliferation (Laville *et al.*, 2004). One limitation noted by the authors is the variability of cytosolic acid phosphatase expression between cell types, and across conditions which may influence expression. An assay that directly reflects cell number (such as the MB assay), rather than possible biological indicators of cell number, would have a clear advantage. This methylene blue assay will be very useful for standardizing any biological marker by cell number.

Methylene blue becomes very useful in cases where it is difficult or impossible to obtain a cell count by traditional methods. In particular, cell numbers in a 96-well plate are so low that it is difficult or even impossible to trypsinize and harvest the cells for trypan blue counting or protein extraction. Methylene blue eliminates the need for removing the cells from the wells, and has been shown to provide an accurate cell count where trypan blue counting, for example, is unsatisfactory (Figures 2.3 and 2.4).

The cell proliferation assay illustrated in Figure 2.6 is an example of a useful food chemistry application of this method. The standard curve  $r^2$  of 0.9949 demonstrates that absorbance is directly proportional to cell number. The data in Figure 2.6a and b clearly show that apple extract inhibited proliferation of MCF-7 cells after 72 hours of treatment by showing the change in actual cell numbers. This cell based assay has many useful applications in phytochemical research, and can be used to test the effects of a variety of whole food extracts or phytochemicals on any cell line.

The modifications made to current methylene blue assays are simple yet valuable. We have demonstrated that methylene blue standard curves of optical density versus cell number produce  $r^2$  values  $>0.99$  (Figure 2.3). Such standard curves can be used to convert methylene blue absorbance of samples in any culture plate to an accurate cell number. Compared to traditional trypan blue cell counting on a hemacytometer, the results from the methylene blue assay are more accurate and more reliable (Figure 2.4). The methylene blue assay has clear advantages over traditional methods for several applications, including cell counting (eg. growth curves, Figure 2.5) and cytotoxicity assays. This method of direct cell counting is reliable and inexpensive, enabling its wide use in the research areas of natural products, bioactive compounds, phytochemicals, functional foods and nutraceuticals. The methylene blue assay modified here is a powerful tool for any application requiring a versatile, efficient, and accurate method of cell counting.

## REFERENCES

- Chu, Y.F., J. Sun, X. Wu and R.H. Liu. 2002. Antioxidant and antiproliferative activities of common vegetables. *Journal of Agricultural and Food Chemistry*. 50: 6910-6916.
- Dewanto, V., X.Z.Wu, K.A. Kafui, and R.H. Liu. 2002. Thermal processing enhances the nutritional value of tomatoes by increasing total antioxidant activity. *Journal of Agricultural and Food Chemistry*. 50: 3010-3014.
- Eberhardt, M.V., C.Y. Lee and R.H. Liu. 2000. Antioxidant activity of fresh apples. *Nature*. 405: 903-904.
- Evans, T., E.T. Rosenthal, J. Youngbloom, D. Distel and T. Hunt. 1983. Cyclin: A protein specified by maternal mRNA in sea urchin eggs that is destroyed at each cleavage division. *Cell*. 33: 389-396.
- Finlay, G.J., B.C. Baguley and W.R. Wilson. 1984. A semiautomated microculture method for investigating growth effects of cytotoxic compounds on exponentially growing carcinoma cells. *Analytical Biochemistry*. 139: 272-277.
- Glutzer, M., A.W. Murray and M.W. Kirschner. 1991. Cyclin is degraded by the ubiquitin pathway. *Nature*. 349: 132-138.
- He, X.J. and R.H. Liu. 2007. Triterpenoids isolated from apple peels have potent antiproliferative activity and may be partially responsible for apple's anticancer activity. *Journal of Agricultural and Food Chemistry*. 55: 4366-4370.
- King, R.W., R.J. Deshaies, J-K. Peters and M.W. Kirschner. 1996. How proteolysis drives the cell cycle. *Science*. 274: 1652-1659.
- Lagneau, A., M. Martin, F. Martin and M.F. Michelm. 1977. Cytotoxic effect of peritoneal macrophages on an intestinal carcinoma in rats. Demonstration of a new cytotoxicity test. *Comptes Rendus des Seances de la Societe de Biologie et de ses Filiales*. 171: 90-93.

- Laville, N., S. Ait-Aissa, E. Gomez, C. Casellas and J.M. Porcher. 2004. Effects of human pharmaceuticals on cytotoxicity, EROD activity and ROS production in fish hepatocytes. *Toxicology*. 196: 41-55.
- Liu, C-S, R.P. Glahn and R.H. Liu. 2004. Assessment of carotenoid bioavailability of whole foods using a Caco-2 cell culture model coupled with an in vitro digestion. *Journal of Agricultural and Food Chemistry*. 52: 4330-4337.
- Liu, M, X.Q. Li, C. Weber, C.Y. Lee, J. Brown and R.H. Liu. 2002. Antioxidant and antiproliferative activities of raspberries. *Journal of Agricultural and Food Chemistry*. 50: 2926-2930.
- Liu, Q., F. Shang, W. Guo, M. Hobbs, P. Valverde, V. Reddy and A. Taylor. 2004. Regulation of the ubiquitin proteasome pathway in human lens epithelial cells during the cell cycle. *Experimental Eye Research*. 78: 197-205.
- Liu, R.H., J.R. Jacob, J.H. Hotchkiss, P.J. Cote, J.L. Gerin and B.C. Tennant. 1994. Hepatitis virus surface antigen induces nitric oxide synthesis in hepatocytes: possible role in hepatocarcinogenesis. *Carcinogenesis*. 15: 2875-2877.
- Liu, R.H. and J. Sun. 2003. Antiproliferative activity of apples is not due to phenolic-induced hydrogen peroxide formation. *Journal of Agricultural and Food Chemistry*. 51: 1718-1723.
- Lowry, O.H., N.J. Rosenbrough, A.L. Farr and R.J. Randall. 1951. Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry*. 193: 265-275.
- Mosmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*. 65(1-2): 55-63.
- Oliver, M.H., N.K. Harrison, J.E. Bishop, P.J. Cole and G.J. Laurent. 1989. A rapid and convenient assay for counting cells cultured in microwell plates: application for assessment of growth factors. *Journal of Cell Science*. 92: 513-518.
- Shirane, M., Y. Harumiya, N. Ishida, A. Hirai, C. Miyamoto, S. Hatakeyama, K. Nakayama and M. Kitagawa. 1999. Down-regulation of p27Kip1 by two

mechanisms, ubiquitin-mediated degradation and proteolytic processing. *Journal of Biological Chemistry*. 274(20): 13886-13893.

Sun, J., Y.F. Chu, X. Wu and R.H. Liu. 2002. Antioxidant and antiproliferative activities of common fruits. *Journal of Agricultural and Food Chemistry*. 50: 7449-7454.

Sun, J. and R.H. Liu. 2006. Cranberry phytochemical extracts induce cell cycle arrest and apoptosis in human MCF-7 breast cancer cells. *Cancer Letters*. 241: 124-134.

Wolfe, K.L. and R.H. Liu. 2007. Cellular antioxidant activity (CAA) assay for assessing antioxidants, foods, and dietary supplements. *Journal of Agricultural and Food Chemistry*. 55: 8896-8907.

Yang, T-T., P. Sinai and S.R. Kain. 1996. An acid phosphatase assay for quantifying the growth of adherent and nonadherent cells. *Analytical Biochemistry*. 241: 103-108.

Yoon, H. and R.H. Liu. 2007. Effect of selected phytochemicals and apple extracts on NF- $\kappa$ B activation in human breast cancer MCF-7 cells. *Journal of Agricultural and Food Chemistry*. 55: 3167-3173.



## CHAPTER 3

### QUERCETIN-3- $\beta$ -D-GLUCOSIDE INHIBITS IGF-I-MEDIATED MCF-7 CELL SURVIVAL BY BLOCKING CELL CYCLE PROGRESSION AND INDUCING APOPTOSIS VIA DOWN-REGULATION OF AKT ACTIVATION

#### ABSTRACT

In the United States, breast cancer is the most frequently occurring cancer among women, accounting for an estimated 26% of new diagnoses in 2008, and is the second-leading cause of cancer deaths among women, accounting for 15% of cancer deaths estimated in 2008. An estimated 30% of cancer risk may be attributed to diet. Epidemiological evidence supports the association between fruit and vegetable consumption and reduced risk of developing cancer. In particular, apple consumption has been correlated with reduced breast cancer risk as well as more favorable prognostic factors. Our lab has previously shown that whole apple extracts inhibited human breast cancer cell proliferation *in vitro* and mammary cancer *in vivo*. IGF-I Receptor signaling may be as critical as Estrogen Receptor signaling in breast cancer. The objective of this study was to determine whether whole apple extracts and purified phytochemicals from apples interfered with IGF-I-mediated signal transduction pathways in MCF-7 human breast cancer cells. Quercetin-3- $\beta$ -D-Glucoside (Q-3-G) proved to be surprisingly effective, inhibiting IGF-I-induced MCF-7 cell proliferation more potently than FBS-stimulated proliferation, as well as more potently than quercetin inhibited cell growth. Q-3-G blocked cell cycle progression and induced apoptosis by interfering with the PI3K pathway; namely, down-regulation of Akt phosphorylation and Cyclin D1 expression. The results of this study help to explain

the mechanisms by which apple consumption may reduce breast cancer risk. Additionally, such studies of purified compounds contribute to the understanding of cancer cell behavior and may inform future endeavors in chemotherapeutic drug design.

## INTRODUCTION

In the United States, breast cancer is the most frequently occurring cancer among women, accounting for an estimated 26% of new diagnoses in 2008, and is the second-leading cause of cancer deaths among women, accounting for 15% of cancer deaths estimated in 2008 (estimates exclude basal and squamous cell skin cancers and *in situ* carcinoma except bladder) (American Cancer Society, 2008). The American Cancer Society predicts ~185,000 new cases and more than 40,000 deaths due to breast cancer in 2008. While advances in treatment are being made, cancer is still considered a preventable disease. Therefore, research in the area of breast cancer prevention is necessary and valuable.

An estimated 30% of cancer risk may be attributed to diet (Willett, 1995; Peto, 2001). Epidemiology studies have consistently demonstrated the correlation between diets high in fruits, vegetables, and whole grains, and reduced risk of developing cancer (Block *et al.*, 1992). Apples, in particular, have been associated with decreased breast cancer risk. A multi-center case-control study in Italy found that, controlling for total fruit consumption, patients who consumed  $\geq$  one apple per day had an odds ratio of breast cancer incidence of 0.82 compared to those who ate fewer than one apple per day (Gallus *et al.*, 2005). Our lab has shown that apples fed to Sprague-Dawley rats in doses equivalent to human consumption of 1, 3 or 6 apples per day significantly reduced tumor incidence, size and burden and delayed onset of DMBA-induced

mammary tumors in a dose-dependent manner (Liu *et al.*, 2005). Our lab has also shown that whole apple extracts inhibit MCF-7 cell cancer growth *in vitro* (Yoon and Liu, 2007; Sun and Liu, 2008; Felice *et al.*, 2009).

IGF-I (Insulin-like Growth Factor-I) is a hormone which acts as a mediator between growth hormone (GH) and growth during fetal and early childhood development (Holly *et al.*, 1999). IGF-I also has the potential to support neoplastic growth. Circulating levels of IGF-I, controlling for the IGFBP3 binding protein, have been shown to correlate more significantly with hormone responsive breast cancer than circulating levels of estrogen, controlling for SHBG (sex hormone binding globulin) (Holly *et al.*, 1999). In premenopausal women, IGF-I/IGFBP3 levels correlate with high mammographic breast density (Byrne *et al.*, 2000), which in turn is known to correlate with increased breast cancer risk (Hankinson *et al.*, 1998). While targeting the ER in estrogen-responsive breast cancers has been largely successful, targeting the IGF-I Receptor (IGF-IR) offers as much if not more potential to prevent and treat breast cancer.

The IGF-IR signals through two major pathways: the PI3K/Akt pathway, and the Mek/Erk MAPK pathway (Werner and LeRoith, 2000). In MCF-7 cells, the IGF-IR exerts its mitogenic effects through the PI3K/Akt pathway (Dufourny *et al.*, 1997), which was the focus of this study. Binding of IGF-I to the IGF-IR stimulates autophosphorylation of the receptor which then phosphorylates Insulin Receptor Substrate-1 (IRS-1; Izumi *et al.*, 1987; Shemer *et al.*, 1987). Phosphorylation of IRS-1 creates a docking site suitable for Src-homology-domain-containing signaling proteins such as the p85 subunit of PI3K. Through phosphorylation of precursor molecules in the lipid membrane, PI3K is responsible for the generation of phosphatidylinositol-3, 4, 5-triphosphate (PIP3), which recruits other molecules such as PDK-1 and Akt. PDK-1 is then able to phosphorylate and activate Akt, which is responsible for

activating a myriad of downstream signaling pathways (Lawlor and Alessi, 2001; Cantley, 2002). As a result of IGF-IR signaling, Akt drives cell cycle progression by promoting transcription of Cyclin D1 as well as preventing Cyclin D1 degradation by inhibiting GSK-3 (Lawlor and Alessi, 2001). Akt suppresses apoptosis by phosphorylating and thus inhibiting the pro-apoptotic mitochondrial protein Bad (Downward, 1999), and phosphorylating and inactivating Caspase-9 (Cardone *et al.*, 1998).

The objective of this study was to determine the effects of two phytochemicals found in apples, quercetin and quercetin-3- $\beta$ -D-glucoside (Q-3-G), on IGF-I-stimulated MCF-7 cell growth *in vitro*. We found that Q-3-G very potently and specifically inhibited MCF-7-cell growth by altering cell cycle distribution and inducing mass apoptosis via down-regulation of Akt phosphorylation.

## MATERIALS AND METHODS

**Reagents.**  $\alpha$ -Minimal Essential Medium ( $\alpha$ -MEM), phenol-red free  $\alpha$ -MEM, fetal bovine serum (FBS), Hank's Balanced Salt Solution (HBSS) and Dulbecco's Phosphate Buffered Saline (PBS) were purchased from Gibco Life Technologies (Grand Island, NY). Methylene blue was purchased from BioQuest (Bedford, MA), ethanol from Mallinckrodt (Phillipsburg, NJ), and acetic acid from Fisher Scientific (Pittsburgh, PA). Quercetin, quercetin-3- $\beta$ -D-glucoside (Q-3-G), glutaraldehyde, transferrin, bovine serum albumin (BSA), propidium iodide (PI), Triton X-100, acridine orange (AO) and ethidium bromide (EB) were purchased from Sigma-Aldrich (St. Louis, MO). Recombinant human IGF-I was purchased from R&D Systems (Minneapolis, MN).

**Antibodies.** The primary antibodies (anti-IGF-I-Receptor  $\beta$ , anti-Phospho-IGF-I-Receptor  $\beta$  [Tyr<sup>1135/1136</sup>], anti-Akt, anti-Phospho-Akt [Ser<sup>473</sup>], anti-Cyclin D1, anti-CDK-4, anti-PCNA, anti-Bcl-2, anti-p27, anti-Caspase-9 and anti-PARP) and the secondary antibodies (anti-Mouse IgG, anti-Rabbit IgG, and anti-Biotin) were purchased from Cell Signaling Technology (Danvers, MA). Anti- $\beta$ -actin and anti-p21 were purchased from Sigma (St. Louis, MO).

**Cell Culture.** MCF-7 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD). For culture maintenance and experiments requiring FBS,  $\alpha$ -MEM containing 10 mM Hepes, 50 units/mL penicillin, 50  $\mu$ g/mL streptomycin, 100  $\mu$ g/mL gentamicin, and 10% FBS was used (referred to as FBS). Cells were kept in a humidified incubator at 37°C with 5% CO<sub>2</sub> and were passaged every 5 days (Liu and Sun, 2003). For starvation purposes and for all experiments using IGF-I, phenol-red free, serum-free  $\alpha$ -MEM, containing 0.2 mg/mL bovine serum albumin (BSA), 10  $\mu$ g/mL transferrin, 10 mM Hepes, 50 units/mL penicillin, 50  $\mu$ g/mL streptomycin, and 100  $\mu$ g/mL gentamicin, was used (referred to as Serum Free Medium, or SFM) (Dufourny *et al.*, 1997).

**Apple Extraction.** Whole apple extracts were prepared using the method previously described by our laboratory (Sun *et al.*, 2002). Briefly, whole apples of the Red Delicious variety were cleaned, dried, and cored. Apples were homogenized in ice-cold 80% acetone for 5 minutes in a chilled Waring blender followed by 3 minutes in a Polytron homogenizer. After vacuum filtration through a no. 2 Whatman filter paper on a Buchner funnel, the filtrate was evaporated at 45°C to reduce the volume by at least 90%. Filtrate was brought up to volume with dH<sub>2</sub>O and stored at -40°C until use.

**Cytotoxicity.** MCF-7 cells were plated at a density of  $4 \times 10^4$  cells per well and incubated to attach to the plates. Cells were then serum-starved for 12 hours and treated for 24 hours with varying doses of phytochemicals (quercetin or Q-3-G) in either  $\alpha$ -MEM with 10% FBS or in serum-free and phenol red-free  $\alpha$ -MEM with 100 ng/mL IGF-I. All Q-3-G treatments contained 1% methanol (MeOH), while all quercetin treatments contained 0.5% dimethyl sulfoxide (DMSO). Cell numbers were determined by the methylene blue assay as described previously (Felice *et al.*, 2009). Briefly, medium was gently removed from the cells and each well was rinsed once with 100  $\mu$ L PBS, and 50  $\mu$ L methylene blue staining solution (HBSS + 1.25% glutaraldehyde + 0.6% methylene blue) were added to each well. The plates were returned to the 37°C incubator for 1 hour. After removal of the methylene blue, the plates were carefully rinsed 3 times in dH<sub>2</sub>O. 100  $\mu$ L of elution solution (50% ethanol + 49% PBS + 1% acetic acid) were added to each well and incubated for 15 minutes at room temperature with gentle agitation. Absorbance was read on an automated plate reader (Dynex Technologies, Chantilly, VA) at a wavelength of 570 nm. Doses which contained  $\geq 90\%$  cell viability compared to the respective control (FBS or IGF-I plus solvent) were considered non-cytotoxic. Values are presented as percent of control, mean  $\pm$  S.D., at least in triplicates.

**Cell proliferation.** To determine effects on cell proliferation, MCF-7 cells were plated in 96-well plates at a density of  $2.5 \times 10^4$  cells per well, incubated for 24 hours, serum-starved for 12 hours, and treated for 72 hours. Medium containing the appropriate treatments was refreshed after the first 48 hours of treatment. Cells were treated with various combinations of FBS or IGF-I with whole apple extracts (contained no solvent), 2- $\alpha$ -hydroxy-ursolic acid (0.1% DMSO), Q-3-G (1% MeOH) or quercetin (0.5% DMSO), in doses shown in Figures 3.1 and 3.2. Cell numbers were

determined using the methylene blue assay described above, and are presented as percent of the control, mean  $\pm$  S.D., at least in triplicates.

**Cellular Antioxidant Activity (CAA) Assay.** The CAA Assay, developed in our lab (Wolfe and Liu, 2007), measures antioxidant activity of pure phytochemicals in a cell culture model. Briefly, cells are treated with a combination of the phytochemical being tested and dichlorofluorescein diacetate (DCFH-DA), the substrate. Once inside the cell, the non-polar DCFH-DA is deacetylated to the more polar dichlorofluorescein (DCFH). Peroxyl radicals generated from 2,2'-azobis(2-amidinopropane) dihydrochloride (ABAP) lead to the oxidation of DCFH to form a polar fluorescent compound, dichlorofluorescein (DCF). The effects of phytochemicals that quench the peroxyl radicals and prevent the oxidation of the DCFH to DCF are measured as a decreased fluorescence. MCF-7 cells were plated in 96-well plates and incubated for 24 hours. After medium was removed, cells were washed with PBS and treated in triplicate wells for 1 h with  $\alpha$ -MEM containing 25  $\mu$ M DCFH-DA in addition to varying doses of either quercetin or Q-3-G. 600  $\mu$ M ABAP in HBSS was added to each well, and plates were read on a Fluoroskan Ascent FL plate-reader (ThermoLabsystems, Franklin, MA) at 5 minute increments for a duration of 1 hour (emission at 538 nm and excitation at 485 nm). Results were quantified as described previously (Wolfe and Liu, 2007; Wolfe *et al.*, 2008) and are presented as an EC<sub>50</sub> for cellular antioxidant activity.

**Flow Cytometry.** The flow cytometry analysis was conducted using the method described previously (Sun and Liu, 2008). Briefly, MCF-7 cells were plated in 6-well plates at a density of  $5 \times 10^5$  cells per well and incubated for 12 hours, after which the cells were serum-starved in SFM for 12 hours. Cells were treated in SFM containing 100 ng/mL IGF-I, 0.15% MeOH, and varying doses of Q-3-G as shown in

Figure 3.3. Samples were harvested at 24 and 48 hours, stained in a detergent hypotonic propidium iodide solution (0.1% sodium citrate, 0.05 mg/mL propidium iodide, 0.1% v:v Triton-X 100), and stored on ice in the dark at 4°C (Yen *et al.*, 1998; Sun and Liu, 2008). Samples were filtered through a 40 micron nylon mesh and run on a BD Biosciences LSRII flow cytometer (San Jose, CA), with collection of the PI emission in a 576/26 band pass filter. Cell cycle and sub-G0 data were analyzed with BD FACSDiva software (Sun and Liu, 2008). Results are presented as percent of events in the parent population, where the parent population for the cell cycle analysis was the total number of singlet intact nuclei in the sample and the parent population for the sub-G0 peak analysis was the total number of singlet events in the sample; mean  $\pm$  S.D, n = 3 except in the 24 hour SFM control where n = 2.

**Western blotting.** The Western blotting protocol was described previously (Liu *et al.*, 1997) Briefly, MCF-7 cells were plated in 6-well plates at a density of  $5 \times 10^5$  cells per well, incubated for 24 hours, serum-starved for 12 hours, and treated with SFM, IGF-I, or IGF-I with Q-3-G for the times indicated in the figure legends. All treatments and controls contained 0.2% MeOH. Cells were harvested by scraping in ice-cold PBS, centrifuged, and re-suspended in RIPA lysis buffer (containing 50 mM Tris, pH 7.4; 1% Igepal; 150 mM sodium chloride; 1mM EDTA) with protease inhibitors (1  $\mu$ g/mL aprotinin; 1  $\mu$ g/mL leupeptin; 1  $\mu$ g/mL pepstatin; 1 mM phenylmethylsulfonyl fluoride [PMSF]; 1 mM sodium orthovanadate; 1 mM sodium fluoride). Total protein concentration was determined using Sigma Total Protein Kit (Sigma-Aldrich) and a Dynex Microplate Reader (Dynex Technologies, Chantilly, VA). Samples were boiled with Laemmli 2 $\times$  sample buffer for 5 minutes, separated on 12% SDS-polyacrylamide gels and transferred to Immobilon-p membranes (Milipore, Billerica, MA) as described previously (Liu *et al.*, 1997). Membranes were blotted



with the appropriate primary antibody (anti-phospho-IGF-I Receptor, anti-Akt, anti-phospho-Akt, anti-Cyclin D1, anti-CDK-4, anti-PCNA, anti-PARP, anti-Caspase-9, or anti-p21) followed by incubation with the appropriate secondary antibody (anti-mouse or anti-rabbit IgG HRP-conjugate). The LumiGLO chemiluminescent reagent and hydrogen peroxide reagent (Cell Signaling Technology, Danvers, MA) were applied before exposing membranes to Kodak Biomax MR Film (Kodak, Rochester, NY) film. The phospho-IGF-IR membranes were stripped of the antibodies (30 minutes on shaker at 50°C in stripping buffer containing 0.76% Tris base, 2% sodium dodecyl sulfate [SDS] and 0.7%  $\beta$ -mercaptoethanol, pH 6.8) and re-probed for total IGF-IR expression. The Cyclin D1 membranes were stripped and re-probed for Bcl-2 expression. The p21 membranes were stripped and re-probed for p27 expression. Protein expression was determined by calculating the optical density of the bands using Labworks software (UVP Laboratories, Upland, CA) and expression was presented as percent of IGF-I control, mean  $\pm$  S.D, n = 3 except for Akt and p-Akt where n = 6.

**Acridine Orange/Ethidium Bromide (AO/EB) Staining.** The AO/EB staining of cells was measured using a modified method (Cotter and Martin, 1996). MCF-7 cells were serum-starved for 12 hours and treated with 100 ng/mL IGF-I and varying doses of Q-3-G for 24 hours. All treatments and controls contained 0.2% MeOH. To harvest the cells, culture medium was first removed and saved. Cells were removed by trypsinization and were recombined with the medium from the well. Samples were then centrifuged and cells were re-suspended in a solution of 100  $\mu$ g/mL acridine orange and 100  $\mu$ g/mL ethidium bromide in 0.9% saline for 2 minutes. Sample tubes were protected from light, kept on ice, and coded to ensure blinded counting. After 2 minutes, 10  $\mu$ L of sample were placed on a slide for counting under

fluorescence microscopy using a 488 nm illumination/520 nm emission filter. Cells were scored for apoptosis as follows. Live non-apoptotic cells were green, with green nuclei and a normal distribution of chromatin. Early apoptotic cells had condensed chromatin in the form of a crescent shape or as bright beads. The bright nucleus may be green or yellow. Late apoptotic cells had bright orange nucleus, condensed orange chromatin, and reduced cell size. Necrotic cells had orange nuclei with a normal distribution of chromatin, and dead cells were a uniform dark, flat red (Cotter and Martin, 1996; Renvoize *et al.*, 1998; Ribble *et al.*, 2005). Cells were scored and reported as number of cells in each category per 200 cells counted. Results presented are the average of 3 experiments, mean  $\pm$  S.D.

**Statistics.** Statistical analysis was conducted using Minitab Release 15 statistical software (Minitab Inc., State College, PA). For cell proliferation data, values were compared to the control using the student's *t*-test. For cell cycle analysis, a chi-square test was used to determine whether differences existed, followed by an ANOVA/Fisher's LSD for pair-wise comparisons. Western blots and AO/EB staining were also analyzed using ANOVA/Fisher's LSD. Statistical significance was set at  $P < 0.05$  for all analyses.

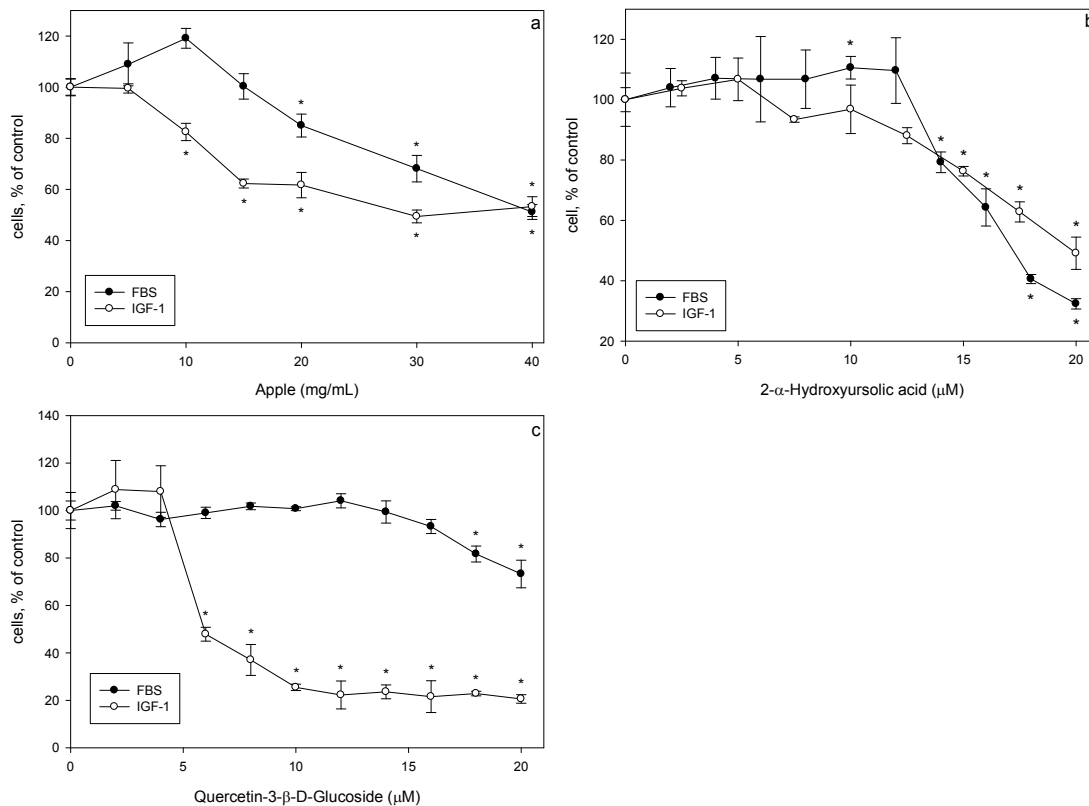
## RESULTS

**Effects of whole apple extracts and pure phytochemicals on IGF-I-induced MCF-7 cell proliferation.** Whole apple extracts inhibited both FBS- and IGF-I-stimulated cell proliferation in a dose-dependent manner (Figure 3.1a). However, the apple extracts were more potent on the IGF-I-treated cells at all doses except for the highest dose of 40 mg/mL, where the proliferation of both the FBS- and IGF-I-

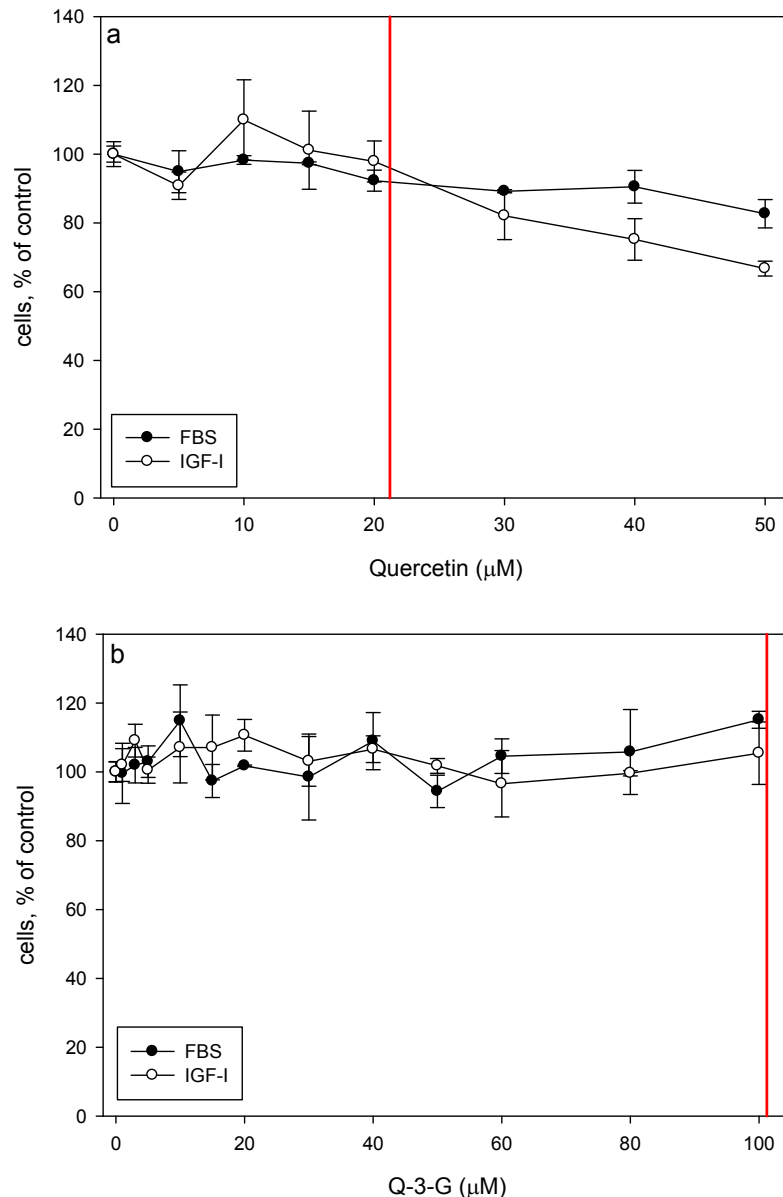
stimulated cells was inhibited by ~50%. Non-cytotoxic doses of 20, 30 and 40 mg/mL whole apple extract reduced FBS-stimulated growth to  $85.0 \pm 4.5$ ,  $68.1 \pm 5.1$ , and  $51.2 \pm 2.9$  % of the FBS control, respectively, while the same doses reduced IGF-I-stimulated growth to  $61.7 \pm 5.0$ ,  $49.4 \pm 2.5$ , and  $53.3 \pm 3.9$  % of the IGF-I control. 2- $\alpha$ -hydroxy-Ursolic Acid (OHUA) inhibited both FBS- and IGF-I-stimulated MCF-7 cell proliferation to a similar extent, indicating the same mechanism of action in both systems (Figure 3.1b). Doses of 14, 16, 18 and 20  $\mu$ M OHUA reduced FBS-induced MCF-7 cell proliferation to  $79.3 \pm 3.4$ ,  $64.3 \pm 6.2$ ,  $40.6 \pm 1.5$  and  $32.4 \pm 1.7$ % of the FBS control, respectively. Doses of 12.5, 15, 17.5 and 20  $\mu$ M OHUA reduced IGF-I-induced MCF-7 cell proliferation to  $88.1 \pm 2.7$ ,  $76.3 \pm 1.6$ ,  $62.8 \pm 3.3$  and  $49.1 \pm 5.3$  % of the IGF-I control, respectively. Quercetin-3- $\beta$ -D-glucoside (Q-3-G) inhibited IGF-I-stimulated cells much more dramatically than FBS-stimulated cells (Figure 3.1c). In FBS-stimulated cells, Q-3-G only significantly inhibited proliferation at doses of 18 and 20  $\mu$ M, reducing proliferation to  $81.7 \pm 3.4$  and  $73.3 \pm 5.8$  % of the FBS control, respectively. However, Q-3-G significantly inhibited IGF-I-induced proliferation at doses as low as 6  $\mu$ M ( $47.9 \pm 2.9$  % of IGF-I control). 8  $\mu$ M Q-3-G reduced proliferation to  $37.0 \pm 6.5$  % of IGF-I control, and 10  $\mu$ M to  $25.5 \pm 1.4$ %. Reduced proliferation began to plateau between 12 and 20  $\mu$ M at around 20% of the IGF-I control. All doses used were non-cytotoxic.

**IGF-I induced MCF-7 cell proliferation.** IGF-I stimulated MCF-7 cell proliferation in a time- and dose-dependent manner (Figure 3.3a and b). After 72 hours treatment,  $\alpha$ -MEM containing 10% FBS increased cell proliferation to  $229.0 \pm 22.4$ % of the cell number at hour zero. 10 ng/mL IGF-I induced cell proliferation to nearly the same extent as the FBS, increasing cell proliferation to  $195.3 \pm 21.2$ % of the cell number at hour zero. The SFM control cell numbers slowly decreased over time to

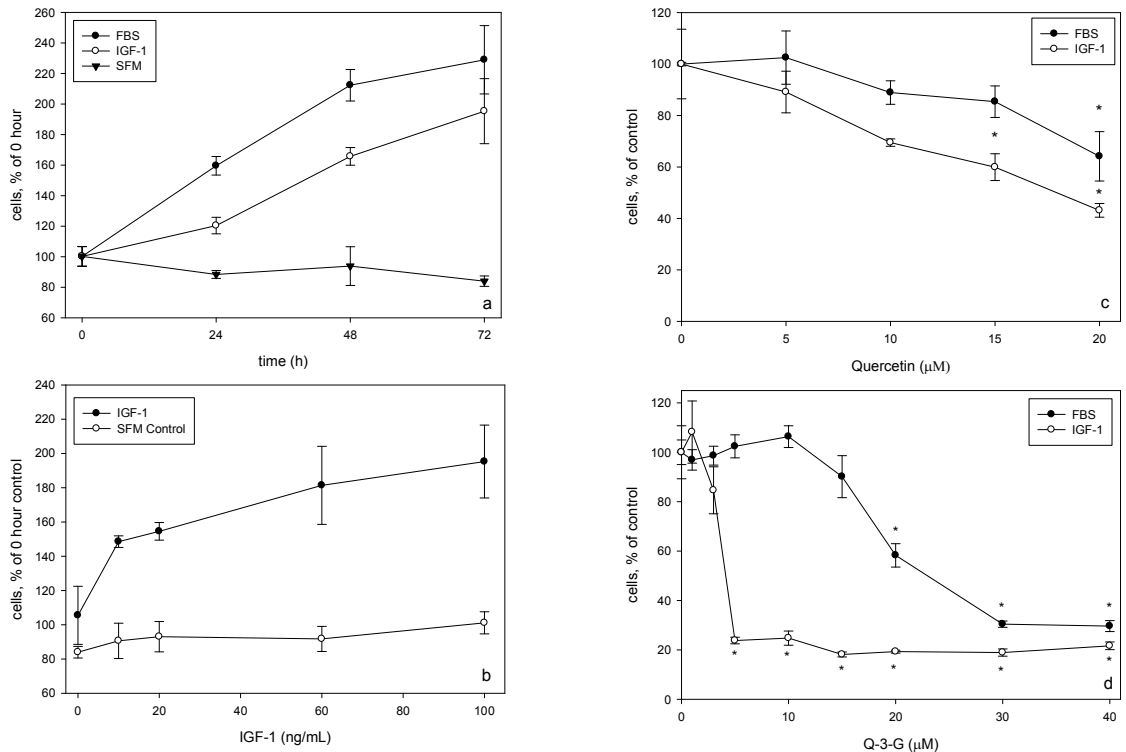
84.0 ± 3.4% of the zero hour cell numbers. Since 100 ng/mL IGF-I stimulated cell proliferation to nearly the same extent as 10% FBS, this dose was used throughout the remaining experiments.



**Figure 3.1. Effects of whole apple extracts and pure phytochemicals on MCF-7 cell proliferation induced by FBS or by IGF-I.** MCF-7 cells were serum-starved for 12 hours, then treated for 72 hours with varying doses of whole apple extracts or pure phytochemical, in  $\alpha$ -MEM with 10% FBS or in serum-free  $\alpha$ -MEM with 10 ng/mL IGF-I. Cell proliferation was measured using the methylene blue assay. **(a) Whole apple extracts.** Apple extracts inhibited both FBS- and IGF-I-stimulated cell proliferation in a dose-dependent manner. However, the apple was more potent against the IGF-I-treated cells. **(b) 2- $\alpha$ -hydroxy-Ursolic Acid (OHUA).** OHUA inhibited both FBS- and IGF-I-stimulated MCF-7 cell proliferation to a similar extent, indicating the same mechanism of action in both systems. **(c) Quercetin-3- $\beta$ -D-glucoside (Q-3-G).** Q-3-G inhibited IGF-I-stimulated cells much more dramatically than FBS-stimulated cells, indicating a mechanism of action involving the IGF-I receptor signaling pathways. (Results are presented as percent of respective controls: mean  $\pm$  SD; n = 3; \*P < 0.05.)



**Figure 3.2. Cytotoxicity of Quercetin and Q-3-G.** MCF-7 cells were serum-starved for 12 hours and incubated for 24 hours with varying doses of the specified phytochemical in  $\alpha$ -MEM containing 10% FBS or 100 ng/mL IGF-I. The methylene blue assay was applied, and viable, attached cells remaining in the wells are presented as percent of the corresponding control. Doses which contained cells in numbers  $\geq 90\%$  of the control were considered non-cytotoxic (indicated by red line). **(a)** In both FBS- and IGF-I-treated cells, quercetin was determined to be non-cytotoxic in doses  $\leq 20 \mu\text{M}$ . **(b)** Quercetin-3- $\beta$ -D-glucoside (Q-3-G) was determined to be non-cytotoxic in doses up to and including  $100 \mu\text{M}$  in both FBS- and IGF-I-treated cells. (Results are presented as percent of respective controls: mean  $\pm$  SD;  $n = 3$ .)



**Figure 3.3. Cell Proliferation.** MCF-7 cells were serum-starved for 12 hours, then treated with various combinations of IGF-I, FBS, quercetin and Q-3-G. Cell numbers were determined by the methylene blue assay. **(a)** MCF-7 cells were treated for 24, 48 and 72 hours with  $\alpha$ -MEM containing either 10% FBS, 100 ng/mL IGF-I, or serum-free  $\alpha$ -MEM (SFM). After 72 hours, 100 ng/mL IGF-I stimulated MCF-7 cell growth to nearly the same extent as did 10% FBS. **(b)** After 72 hours, IGF-I stimulated MCF-7 cell growth in a dose-dependent manner. **(c)** MCF-7 cells were treated for 72 hours with varying doses of quercetin in  $\alpha$ -MEM containing either 10% FBS or 100 ng/mL IGF-I. Quercetin inhibited IGF-I-mediated cell proliferation to a greater extent than FBS-mediated proliferation. **(d)** After 72 hours, Q-3-G inhibited IGF-I-stimulated cell proliferation much more dramatically than FBS-stimulated proliferation, and also much more potently than did quercetin.

**Effects of Quercetin and Q-3-G on FBS- and IGF-I-stimulated MCF-7 cell proliferation.** Non-cytotoxic doses of quercetin (up to 20  $\mu$ mol/L, Figure 3.2a) inhibited IGF-I-stimulated cell proliferation more potently than FBS-stimulated growth (Figure 3.3c). Quercetin (20  $\mu$ mol/L) significantly reduced FBS-stimulated cell proliferation to  $64.1 \pm 9.6\%$  of the FBS control, and significantly reduced IGF-I-stimulated cell proliferation to  $43.1 \pm 2.6\%$  of IGF-I control. Q-3-G inhibited IGF-I-

stimulated MCF-7 cell proliferation much more potently than FBS-stimulated proliferation (Figure 3.3d). Non-cytotoxic doses of Q-3-G (Figure 3.2b) reduced FBS-stimulated cell proliferation to  $58.3 \pm 4.7\%$  of FBS control at 20  $\mu\text{mol/L}$  and plateaued at  $\sim 30\%$  of control at 30  $\mu\text{mol/L}$  ( $30.4 \pm 1.3\%$  at 30  $\mu\text{mol/L}$  and  $29.6 \pm 2.2\%$  at 40  $\mu\text{mol/L}$ ). Q-3-G reduced IGF-I-stimulated proliferation to  $84.6 \pm 9.5\%$  of the IGF-I control at only 3  $\mu\text{mol/L}$ ,  $23.8 \pm 1.4\%$  at 5  $\mu\text{mol/L}$ ,  $24.8 \pm 2.9\%$  at 10  $\mu\text{mol/L}$ , and plateaued at  $\sim 20\%$  beginning at 15  $\mu\text{mol/L}$  ( $18.1 \pm 1.0\%$ ) through 40  $\mu\text{mol/L}$  ( $21.6 \pm 1.5\%$ ); ( $P < 0.05$ ,  $n = 3$ ).

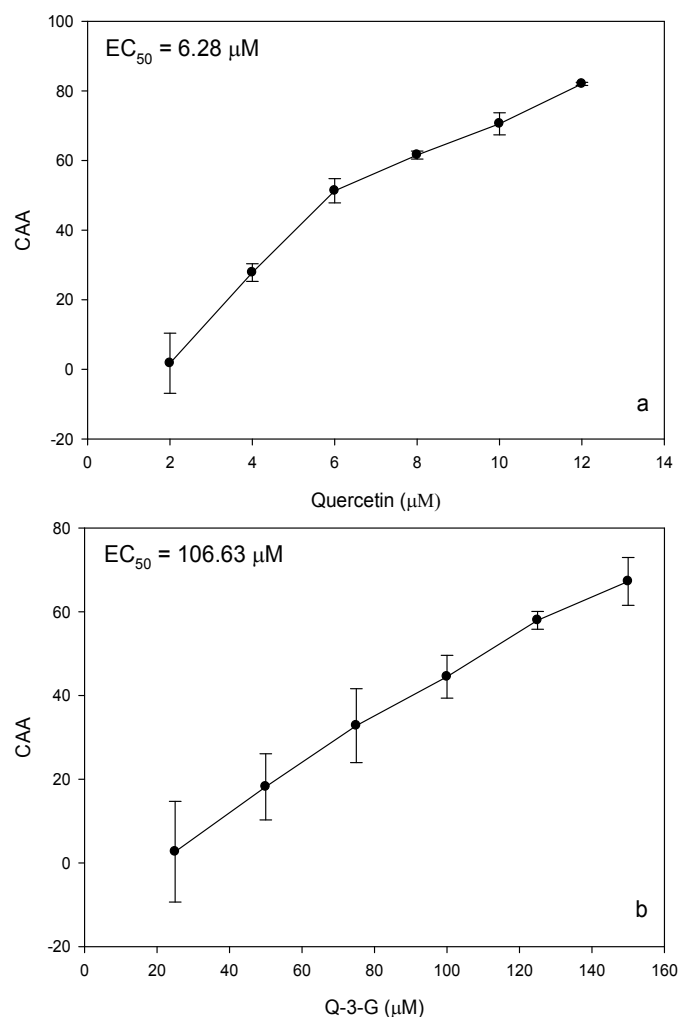
**Q-3-G has weaker cellular antioxidant activity than Quercetin.** MCF-7 cells were treated with either quercetin (0, 2, 4, 6, 8, 10 or 12  $\mu\text{M}$ ) or Q-3-G (0, 25, 50, 75, 100, 125, or 150  $\mu\text{M}$ ) and their cellular antioxidant activities were measured using the CAA assay (Table 3.1 and Figure 3.4). The  $\text{EC}_{50}$  for quercetin was determined to be  $6.28 \pm 0.25 \mu\text{M}$  (Figure 3.4a), while the  $\text{EC}_{50}$  for Q-3-G was  $106.63 \pm 13.26 \mu\text{M}$  (Figure 3.4b).

**Table 3.1. Anti-Proliferative and Anti-Oxidant Activities of Quercetin and Q-3-G in MCF-7 Cells.** *Anti-proliferative Activity.* As seen in Figure 3.1 (c,d), Q-3-G inhibited proliferation of IGF-I-stimulated MCF-7 cells much more dramatically than FBS-stimulated cells. Q-3-G was also much more potent than quercetin was against both IGF-I- and FBS-stimulated cells. *Cellular Antioxidant Activity (CAA) Assay.* MCF-7 cells were treated with phytochemical and the substrate DCFH-DA, followed by the oxidant ABAP. Oxidation of the substrate by ABAP-generated peroxy radicals was measured by a fluorescence plate reader. Quercetin exhibited potent cellular antioxidant activity with an  $\text{EC}_{50}$  of 6.28  $\mu\text{M}$ . Quercetin-3-Glucoside had weak cellular antioxidant activity, with an  $\text{EC}_{50}$  of 106.63  $\mu\text{M}$ . The doses of Q-3-G used in all other experiments in this study (up to 20  $\mu\text{M}$ ) have virtually no antioxidant capacity in MCF-7 cells as determined by the CAA assay.

EC50 ( $\mu\text{M}$ )	Anti-Proliferative Activity		Anti-Oxidant Activity
	FBS	IGF-I	CAA Assay
Quercetin	29.4	17.3	6.3
Q-3-G	24.4	3.6	106.6

**Q-3-G blocked IGF-I-mediated cell cycle progression.** After 24 hours treatment,  $90.1 \pm 0.6\%$  of the cells of SFM control samples were in G0/G1 phase of the cell cycle (Figure 3.5a). Stimulation with 100 ng/mL IGF-I for 24 hours reduced this to  $78.2 \pm 0.2\%$ . Addition of the Q-3-G concomitantly with the IGF-I increased the percentage of G0/G1 cells, in a dose-dependent manner, to a level comparable to that of SFM ( $87.2 \pm 0.8\%$  at 15  $\mu\text{mol/L}$  Q-3-G.) A similar pattern was observed with the S-phase cells in the population (Figure 3.5b).  $5.3 \pm 0.7\%$  of the cells of SFM control were in S phase after 24 hours. Stimulation of IGF-I increased this to  $12.4 \pm 0.5\%$ . Addition of the Q-3-G then reduced the number of S phase cells to  $6.0 \pm 0.2\%$  at 15  $\mu\text{mol/L}$ , similar to the SFM control. Again, the same trend was observed in the G2/M phase cells (Figure 3.5c).  $3.4 \pm 0.1\%$  of the cells of SFM control samples were in G2/M phase. This increased to  $8.2 \pm 0.7\%$  upon IGF-I stimulation, and decreased to  $5.3 \pm 0.4\%$  upon addition of 15  $\mu\text{mol/L}$  Q-3-G. Similar patterns occurred after 48 hours of treatment.  $91.0 \pm 0.8\%$  of the cells of SFM control were in G0/G1 phase,  $82.0 \pm 1.8\%$  of the IGF-I-treated cells were G0/G1, and in samples treated with 15  $\mu\text{M}$  Q-3-G in addition to the 100 ng/mL IGF-I,  $88.5 \pm 0.2\%$  of the population was in G0/G1 phase (Figure 3.5d).  $2.9 \pm 0.3\%$  of the SFM control cells were in S phase,  $9.0 \pm 0.9\%$  of the IGF-I-stimulated cells were in S phase, and cells treated with IGF-I and 5, 10 and 15  $\mu\text{M}$  Q-3-G had  $4.4 \pm 0.2$ ,  $4.4 \pm 0.6$  and  $4.2 \pm 0.2\%$  of their populations in S phase, respectively (Figure 3.5e). Similarly, after 48 hours,  $4.8 \pm 0.7\%$  of the population were G2/M phase cells, which increased to  $7.8 \pm 0.8\%$  after IGF-I stimulation and decreased to  $4.4 \pm 0.8$  and  $4.2 \pm 0.1\%$  after treatment with 10 and 15  $\mu\text{M}$  Q-3-G, respectively (Figure 3.5f).



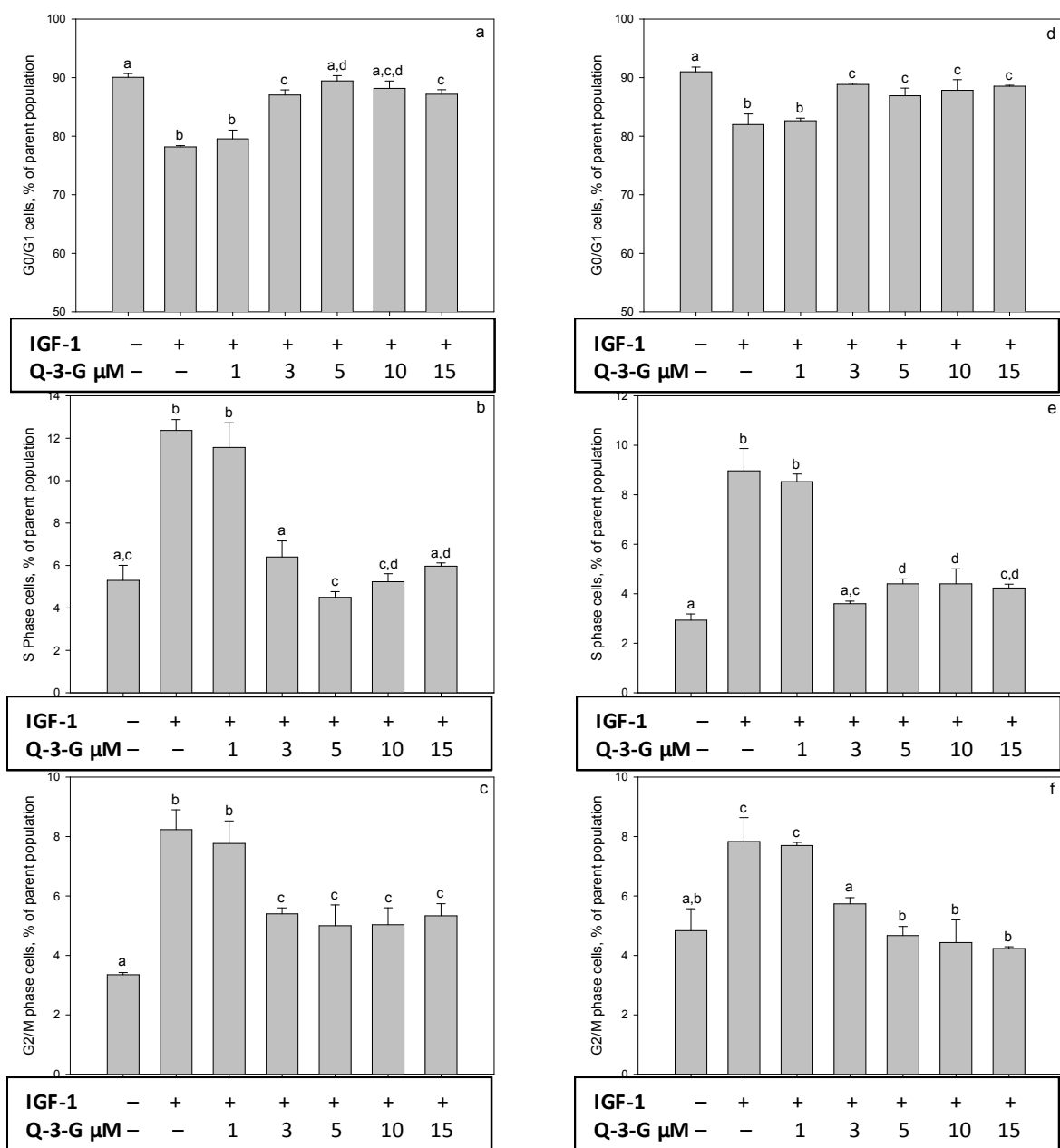


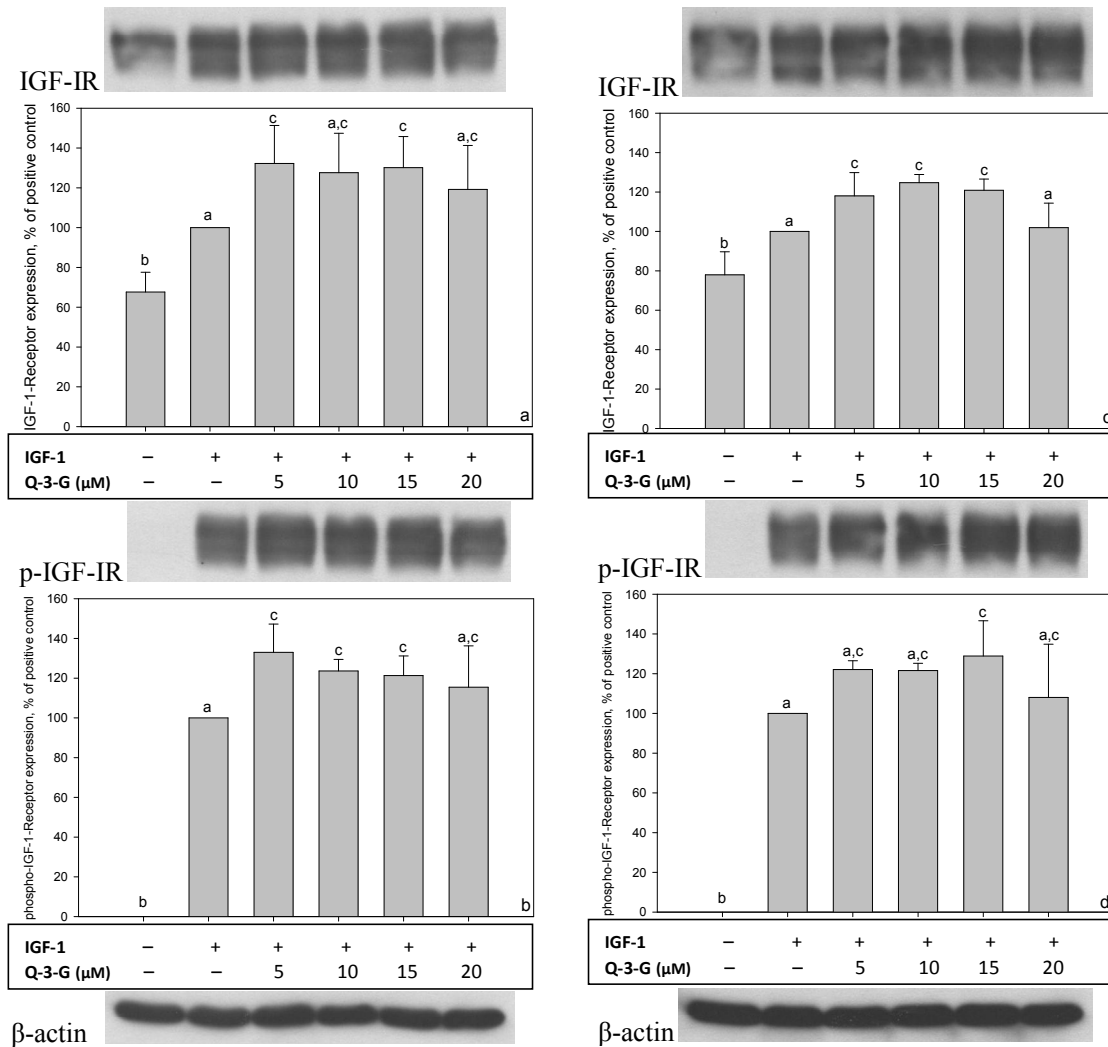
**Figure 3.4. Cellular Antioxidant Activity (CAA) Assay.** MCF-7 cells were treated with phytochemical and the substrate DCFH-DA, followed by the oxidant ABAP. Oxidation of the substrate by ABAP-generated peroxy radicals was measured by a fluorescence plate reader. Quercetin exhibited potent cellular antioxidant activity with an  $\text{EC}_{50}$  of 6.28  $\mu\text{M}$  (a). Quercetin-3-Glucoside had weak cellular antioxidant activity, with an  $\text{EC}_{50}$  of 106.63  $\mu\text{M}$  (b). The doses of Q-3-G used in all other experiments in this study (up to 20  $\mu\text{M}$ ) have virtually no antioxidant capacity in MCF-7 cells as determined by the CAA assay.

### **Q-3-G affected the expression and activation of key cell signaling proteins.**

Effects on IGF-I Receptor expression and activation. After 12 hours treatment, IGF-IR expression under SFM treatment was  $67.6 \pm 9.9\%$  of the IGF-I control. Treatment with Q-3-G in doses of 5, 10, 15 and 20  $\mu\text{mol/L}$  increased IGF-IR expression to  $132.2 \pm 19.1$ ,  $127.6 \pm 19.8$ ,  $130.1 \pm 15.6$ , and  $119.2 \pm 22.1\%$  of the IGF-I control, respectively (Figure 3.6a). The effect of Q-3-G on IGF-IR phosphorylation followed nearly the same trend as that seen in receptor expression (Figure 3.6b), except for the complete absence of IGF-IR phosphorylation in the SFM control, as expected. (IGF-IR phosphorylation, after treatment with 5, 10, 15 and 20  $\mu\text{M}$  Q-3-G in addition to the 100 ng/mL IGF-I, increased to  $133.0 \pm 14.2$ ,  $123.6 \pm 5.8$ ,  $121.3 \pm 9.9$  and  $115.5 \pm 20.8\%$  of the IGF-I control.) A similar pattern was seen after 24 hours of treatment. IGF-IR expression in the SFM control was  $78.0 \pm 11.7\%$  of the IGF-I control, and treatment with 5, 10, 15 and 20  $\mu\text{M}$  Q-3-G increased expression to  $118.0 \pm 11.8$ ,  $124.7 \pm 4.2$ ,  $120.9 \pm 5.7$ ,  $101.9 \pm 12.4\%$  of the IGF-I control, respectively (Figure 3.6c). The IGF-IR phosphorylation followed the same pattern: no phosphorylation in the SFM control, and the 4 doses of Q-3-G increased phosphorylation to  $122.1 \pm 4.4$ ,  $121.6 \pm 3.6$ ,  $128.9 \pm 17.7$ , and  $108.0 \pm 26.8\%$  of the IGF-I control (Figure 3.6c). Therefore, any effects seen on the IGF-IR by Q-3-G appeared to be due to changes in receptor expression rather than phosphorylation.

**Figure 3.5. Effect of Q-3-G on cell cycle distribution of IGF-I-stimulated MCF-7 cells.** MCF-7 cells were serum-starved for 12 hours, then treated for 24 or 48 hours with varying doses of Q-3-G in SFM containing 100 ng/mL IGF-I. Cells were then lysed and stained in a hypotonic propidium iodide staining solution. Nuclei suspensions were passed through a flow cytometer, and cell cycle distribution of the samples was recorded. *G0/G1 phase cells.* After 24 hours of treatment **(a)**, 90.1% of the SFM control parent population (parent population being the singlet nuclei measured in the sample) was in G0/G1 phase of the cell cycle. Stimulation with the IGF-I reduced this to 78.2%. Addition of the Q-3-G along with the IGF-I increased the percentage of G0/G1 cells, in a dose-dependent manner, to a level comparable to that of SFM (>87% at doses of 3 through 15  $\mu$ M). Similar results were seen after 48 hours of treatment **(d)**. *S phase cells.* After 24 hours of treatment **(b)**, 5.3% of the parent population of the SFM control cells were in S phase. Stimulation of IGF-I increased this to 12.4%. Addition of the Q-3-G then reduced the number of S phase cells to levels similar to the SFM control (5.2% at 10  $\mu$ M; 6.0% at 15  $\mu$ M). After 48 hours **(e)**, 2.9% of the SFM control cells were in S phase, 9.0% of the IGF-I-stimulated cells were in S phase, and cells treated with IGF-I and 5, 10 and 15  $\mu$ M Q-3-G had 4.4, 4.4 and 4.2% of their populations in S phase, respectively. *G2/M phase cells.* Again, the same trend was observed in the G2/M phase cells. After 24 hours **(c)**, 3.4% of the SFM control samples were in G2/M phase. This increased to 8.2% upon IGF-I stimulation, and decreased to 5% upon addition of Q-3-G (5.0% at 10  $\mu$ M and 5.3% at 15  $\mu$ M). Similarly, after 48 hours **(f)**, 4.8% of the population were G2/M phase cells, which increased to 7.8% after IGF-I stimulation and decreased to 4.4 and 4.2% after treatment with 10 and 15  $\mu$ M Q-3-G, respectively. (Bars with no letters in common are statistically significantly different,  $P < 0.05$ .)





**Figure 3.6. Effects of Q-3-G on IGF-I Receptor expression and phosphorylation.** MCF-7 cells were plated at a density of  $5 \times 10^5$  cells per well, allowed to attach for 24 hours, and serum-starved for 12 hours. Cells were treated with Q-3-G and IGF-I simultaneously for 12 or 24 hours. After 12 hours, treatment with Q-3-G appeared to increase IGF-IR expression compared to the IGF-I control (**a**). The effect of Q-3-G on IGF-IR phosphorylation (**b**) followed the same trend as that in (a). After 24 hours treatment, Q-3-G in doses of 5 and 10  $\mu$ M appeared to increase IGF-IR expression compared to the IGF-I control, while expression decreased with 15 and 20  $\mu$ M Q-3-G back to a level similar to that of the IGF-I control (**c**). The effect of Q-3-G on IGF-IR phosphorylation (**d**) followed the same trend as that in (c). Therefore, any effects seen on the receptor by Q-3-G appear to be due to changes in expression of the receptor rather than phosphorylation. (Mean  $\pm$  SD,  $n=3$ . Bars with no letters in common are statistically significantly different,  $P < 0.05$ .)

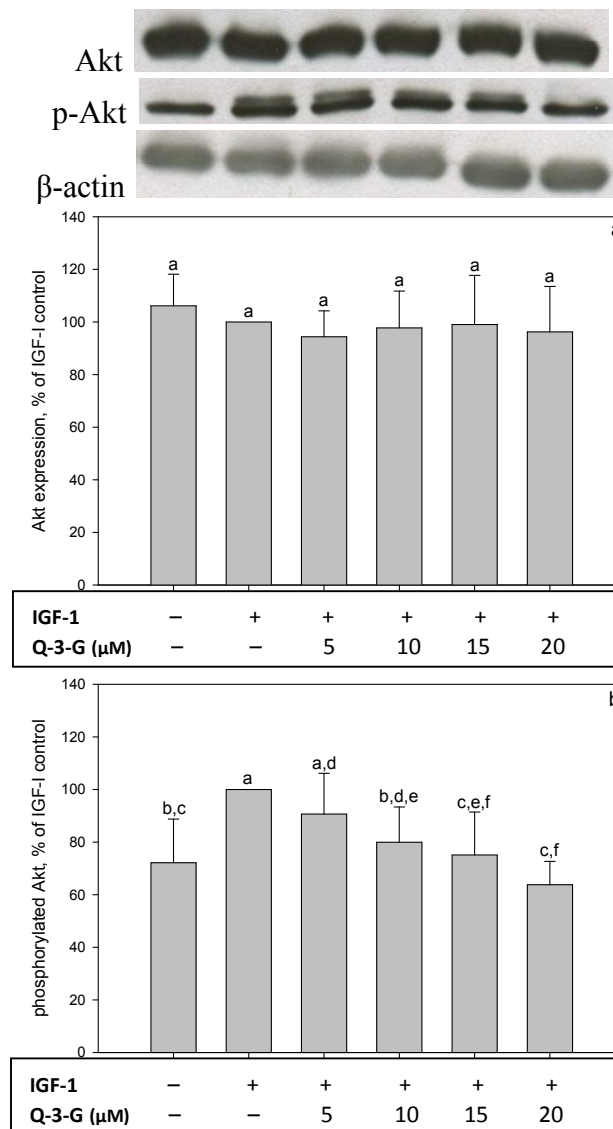
Effects on Akt expression and activation. Neither IGF-I nor Q-3-G affected expression of Akt (Figure 3.7a). However, IGF-I induced Akt phosphorylation (Figure 3.7b) over the SFM control, as expected (Akt phosphorylation in the SFM control was  $72.1 \pm 16.6\%$  of the IGF-I control). Addition of Q-3-G reduced Akt phosphorylation in a clear dose-dependent manner. Doses of 5, 10, 15 and 20  $\mu\text{M}$  Q-3-G reduced Akt phosphorylation to  $90.7 \pm 15.5$ ,  $80.0 \pm 13.4$ ,  $75.1 \pm 16.3$ , and  $63.8 \pm 8.9\%$  of the IGF-I control, respectively (mean  $\pm$  S.D.,  $n=6$ ).

Cyclin D1. After 24 hours of treatment with 100 ng/mL IGF-I, Cyclin D1 expression was clearly up-regulated compared to the SFM control (Figure 3.8). Addition of 5, 10, 15 and 20  $\mu\text{M}$  Q-3-G down-regulated IGF-I-induced Cyclin D1 expression to  $37.3 \pm 12.7$ ,  $31.9 \pm 14.5$ ,  $17.8 \pm 9.7$ , and  $23.4 \pm 8.0\%$  of the IGF-I control, respectively. Q-3-G reversed the effects of IGF-I on Cyclin D1 expression (mean  $\pm$  S.D.,  $n = 3$ ).

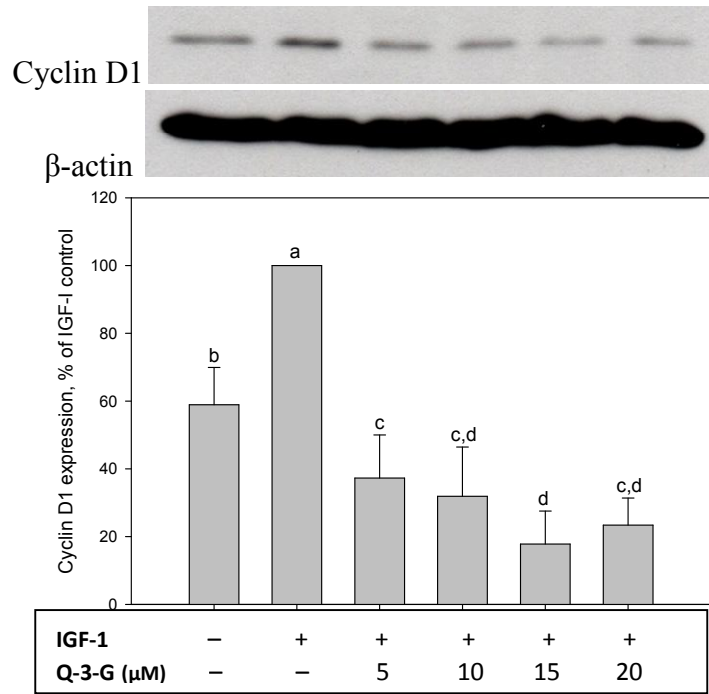
CDK-4. IGF-I treatment upregulated CDK-4 expression (Figure 3.9) when compared to the SFM control ( $77.4 \pm 11.3\%$  of the IGF-I control). Addition of 5, 10, 15 and 20  $\mu\text{M}$  Q-3-G reduced CDK-4 expression to  $58.3 \pm 5.6$ ,  $53.7 \pm 4.5$ ,  $51.5 \pm 10.4$ , and  $63.5 \pm 22.7\%$  of the IGF-I control, respectively. Q-3-G reversed the effects of IGF-I on CDK-4 expression (mean  $\pm$  S.D.,  $n = 3$ ).

p21, p27 and PCNA. The expression of p21, p27 and PCNA were not significantly altered by treatment with IGF-I alone or in combination with various doses of Q-3-G. p21 showed a slight increasing trend upon treatment with IGF-I, and IGF-I with Q-3-G, yet no treatment was significantly different from any other (Figure 3.10a). p27 expression was nearly the same between the SFM and IGF-I controls, and addition of Q-3-G appeared to cause a decreasing trend, although not significantly (Figure 3.10b). Treatment with IGF-I slightly increased PCNA expression over the

SFM control, and addition of Q-3-G decreased the expression, although none of the changes was significant (Figure 3.10c).

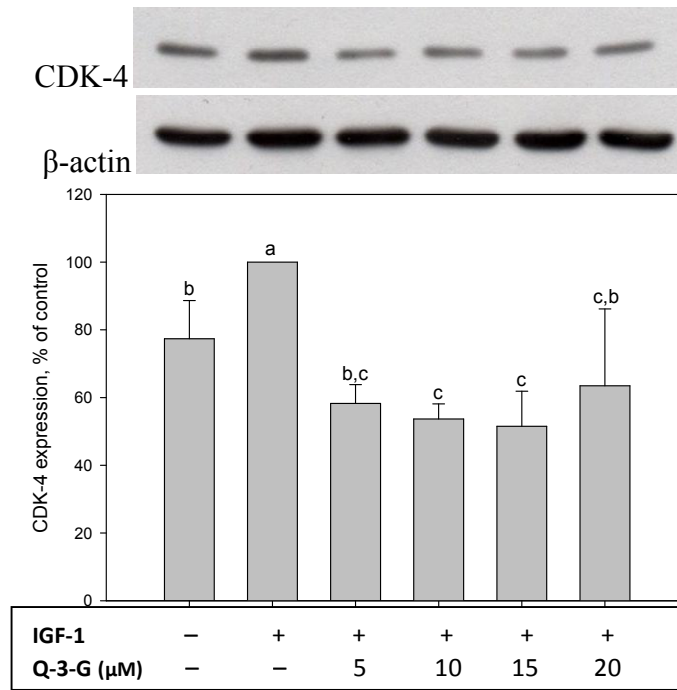


**Figure 3.7. Effects of Q-3-G on Akt expression and phosphorylation. (a) *Akt* expression.** Neither IGF-I nor Q-3-G affected expression of Akt. **(b) *Akt* Phosphorylation.** IGF-I induced Akt phosphorylation over the SFM control, as expected. Addition of Q-3-G to the IGF-I reduced Akt phosphorylation in a clear dose-dependent manner. Doses of 5, 10, 15 and 20  $\mu$ M Q-3-G reduced Akt phosphorylation to  $90.7 \pm 15.5\%$ ,  $80.0 \pm 13.4\%$ ,  $75.1 \pm 16.3\%$ , and  $63.8 \pm 8.9\%$  of the IGF-I control, respectively. (Mean  $\pm$  SD,  $n=6$ . Bars with no letters in common are statistically significantly different,  $P < 0.05$ .)

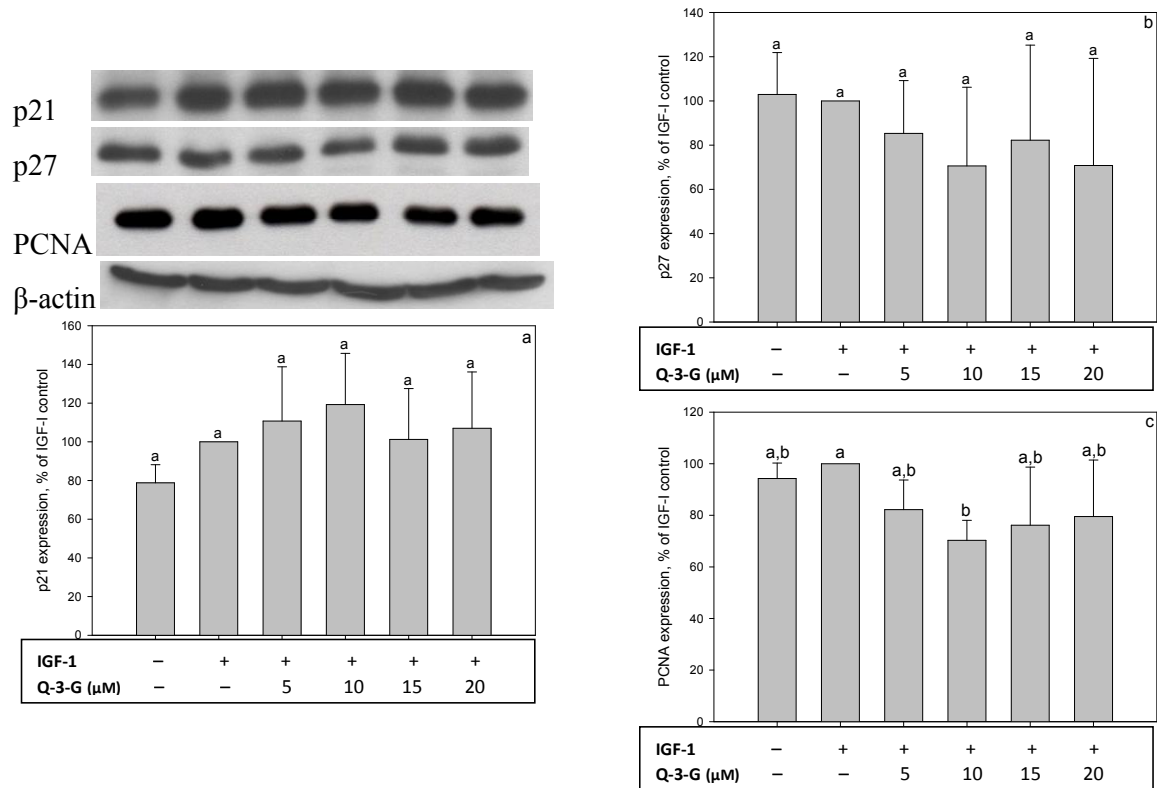


**Figure 3.8. Effect of Q-3-G on Cyclin D1 expression.** MCF-7 cells were plated at a density of  $5 \times 10^5$  cells per well and incubated for 24 hours. Cells were then serum-starved for 12 hours. After 24 hours of treatment with 100 ng/mL IGF-I, Cyclin D1 expression was clearly up-regulated compared to the SFM control. After addition of varying doses of Q-3-G, 5, 10, 15 and 20  $\mu$ M Q-3-G down-regulated Cyclin D1 expression to  $37.3 \pm 12.7\%$ ,  $31.9 \pm 14.5\%$ ,  $17.8 \pm 9.7\%$ , and  $23.4 \pm 8.0\%$  of the IGF-I control, respectively. Q-3-G reversed the effects of IGF-I on Cyclin D1 expression. (Mean  $\pm$  SD, n = 3. Bars with no letters in common are statistically significantly different,  $P < 0.05$ .)





**Figure 3.9. Effect of Q-3-G on CDK-4 Expression.** MCF-7 cells were plated and serum-starved as in Fig. 3.7. Cells were then treated for 24 hours with 100 ng/mL IGF-I and varying doses of Q-3-G. IGF-I treatment upregulated CDK-4 expression compared to the negative control. Addition of 5, 10, 15 and 20  $\mu$ M Q-3-G reduced CDK-4 expression to  $58.3 \pm 5.6\%$ ,  $53.7 \pm 4.5\%$ ,  $51.5 \pm 10.4\%$ , and  $63.5 \pm 22.7\%$  of the IGF-I control, respectively. Q-3-G reversed the effects of IGF-I on CDK-4 expression. (Mean  $\pm$  SD,  $n = 3$ . Bars with no letters in common are statistically significantly different,  $P < 0.05$ .)



**Figure 3.10. Effect of Q-3-G on expression of p21, p27 and PCNA.** MCF-7 cells were plated at a density of  $5 \times 10^5$  cells per well, allowed to attach for 24 hours, and serum-starved for 12 hours. Cells were treated with Q-3-G and IGF-I simultaneously for 24 hours. After 24 hours of treatment, Q-3-G appeared to slightly increase p21 expression (**a**) and slightly decrease p27 expression (**b**), although not significantly. Both IGF-I and Q-3-G had little effect on PCNA expression (**c**). (Mean  $\pm$  SD,  $n=3$ . Bars with no letters in common are statistically significantly different,  $P < 0.05$ .  $\beta$ -actin shown applies to p21 membrane which was stripped and re-probed for p27. PCNA was detected on the same membrane as CDK-4, Fig. 3.8.)

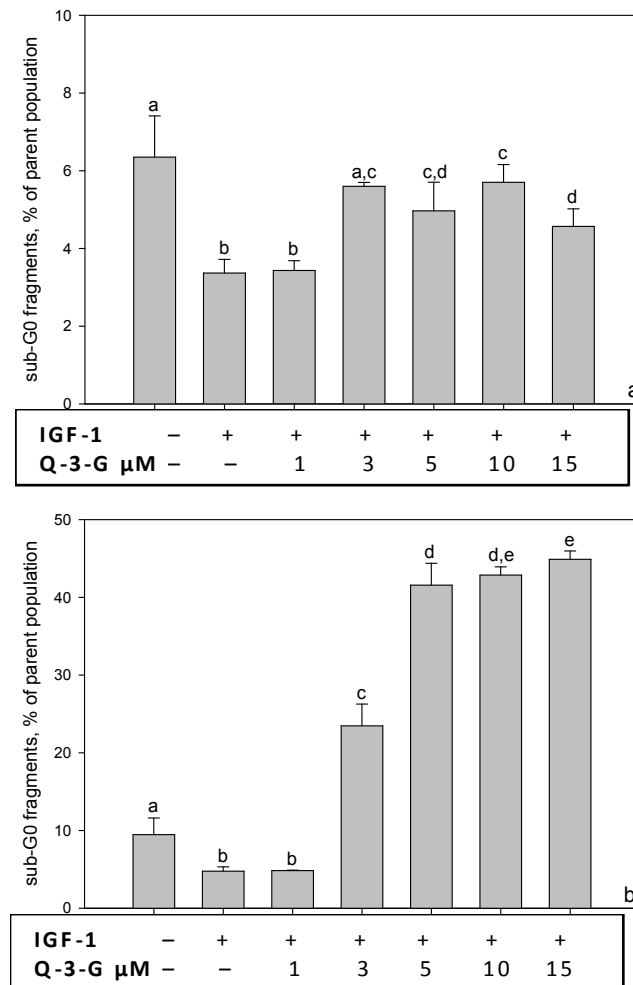
### **Q-3-G reversed IGF-I-mediated apoptosis suppression.**

Flow Cytometry. As expected, addition of IGF-I to the SFM suppressed apoptosis in MCF-7 cells. This was evident in the pattern of sub-G0 fragments seen in the flow cytometric analysis. After 24 hours (Figure 3.11a),  $6.4 \pm 1.1\%$  of the singlet events counted from the SFM control sample were sub-G0 fragments. 100 ng/mL IGF-I reduced this to  $3.4 \pm 0.4\%$ . Addition of Q-3-G reversed the effects of IGF-I, returning the level of sub-G0 fragments to that similar to the SFM control ( $5.7 \pm 0.5\%$  at 10  $\mu\text{M}$  and  $4.6 \pm 0.5\%$  at 15  $\mu\text{M}$ ). Similar results were seen after 48 hrs (Figure 3.11b), only to a much greater extent. In the SFM control sample,  $9.5 \pm 2.1\%$  of the events were sub-G0 fragments, while  $4.8 \pm 0.6\%$  of the IGF-I control events were sub-G0 fragments. With 15  $\mu\text{M}$  Q-3-G,  $44.9 \pm 1.1\%$  of the sample population were sub-G0 fragments, indicating mass cell death through apoptosis.

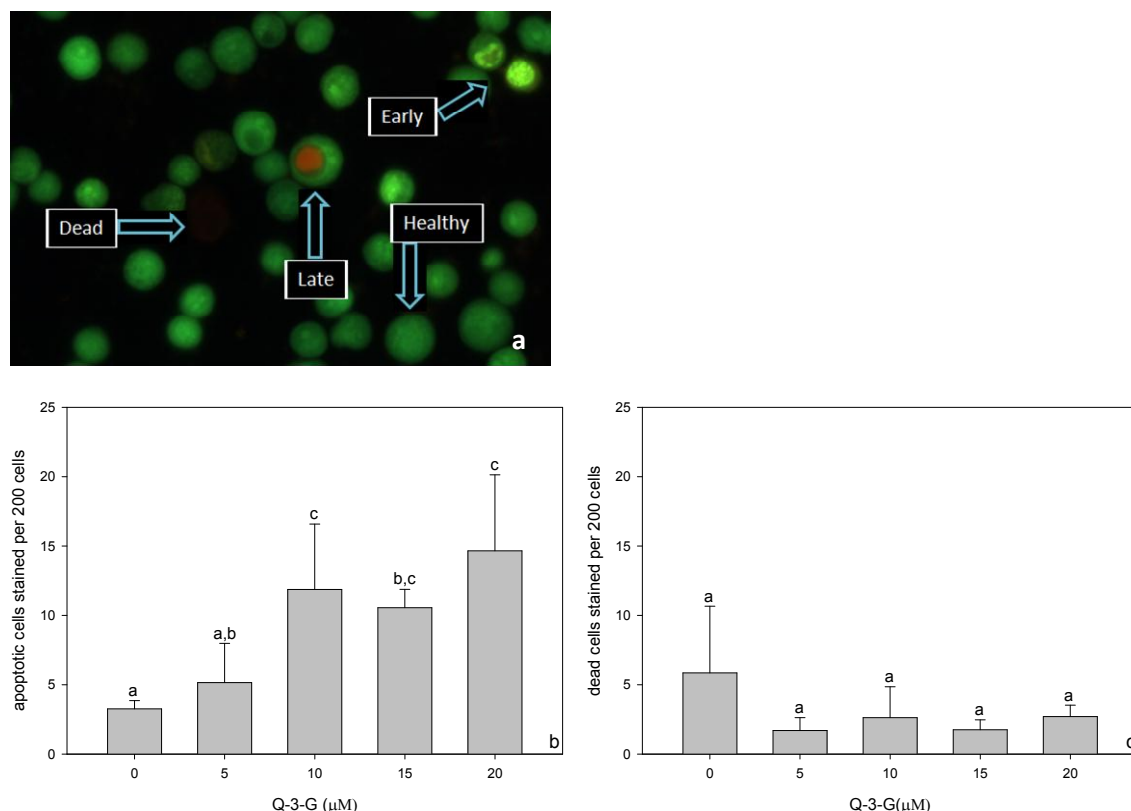
Acridine Orange/Ethidium Bromide (AO/EB) Staining. Figure 3.12a shows AO/EB stained MCF-7 cells. Cells with uniform green appearance were scored as healthy, with bright condensed yellow or orange nuclei as apoptotic, and dark red as dead. All groups were treated with 100 ng/mL IGF-I. Addition of Q-3-G to the IGF-I-treated cells increased the proportion of apoptotic cells in a dose-dependent manner (0  $\mu\text{M}$ :  $3.3 \pm 0.6$  apoptotic cells per 200 cells; 5  $\mu\text{M}$ :  $5.2 \pm 2.8$ ; 10  $\mu\text{M}$ :  $11.9 \pm 4.7$ ; 15  $\mu\text{M}$ :  $10.6 \pm 1.3$ ; 20  $\mu\text{M}$ :  $14.7 \pm 5.5$ , Figure 3.12b). The proportion of dead cells was not significantly different among groups (Figure 3.12c).

Caspase-9 cleavage. Figure 3.13 represents expression of the cleavage fragment of Caspase-9, as a percent of the IGF-I control. There was no significant difference between the SFM and IGF-I controls (SFM:  $93.0 \pm 27.9\%$  of the IGF-I control). However, the administration of Q-3-G in addition to the IGF-I increased Caspase-9 cleavage to  $221.8 \pm 65.5$ ,  $234.2 \pm 57.8$ ,  $248.1 \pm 131.8$ , and  $221.3 \pm 62.4\%$

of the IGF-I control in doses of 5, 10, 15 and 20  $\mu\text{M}$ , respectively. Although the variation was large, all Q-3-G treatment groups were statistically significantly different from the IGF-I control (mean  $\pm$  S.D.,  $n=3$ ,  $p<0.05$ ).

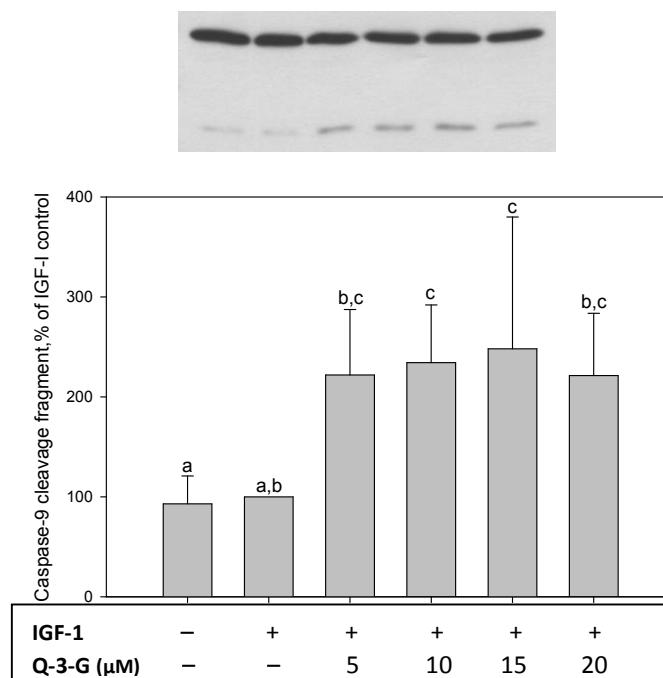


**Figure 3.11. Effect of Q-3-G on Sub-G0 peak.** After 24 hours treatment (a), flow cytometric analysis demonstrated that addition of 100 ng/mL IGF-I reduced the proportion of sub-G0 fragments compared to the SFM control from 6.4% to 3.4% of the parent population (singlet events measured in the sample), indicating the IGF-I suppressed cell death in the MCF-7 cells. Addition of Q-3-G reversed the effects of IGF-I, returning the level of sub-G0 fragments to that similar to the SFM control (4.6% after treatment with 15  $\mu\text{M}$  Q-3-G). Similar results were seen after 48 hrs (b), only to a much greater extent. 9.5% of the SFM control and 4.8% of the IGF-I control were sub-G0 fragments. With 15  $\mu\text{M}$  Q-3-G, more than 44.9% of the parent population were sub-G0 fragments. (Mean  $\pm$  SD,  $n = 3$ . Bars with no letters in common are statistically significantly different,  $P < 0.05$ .)



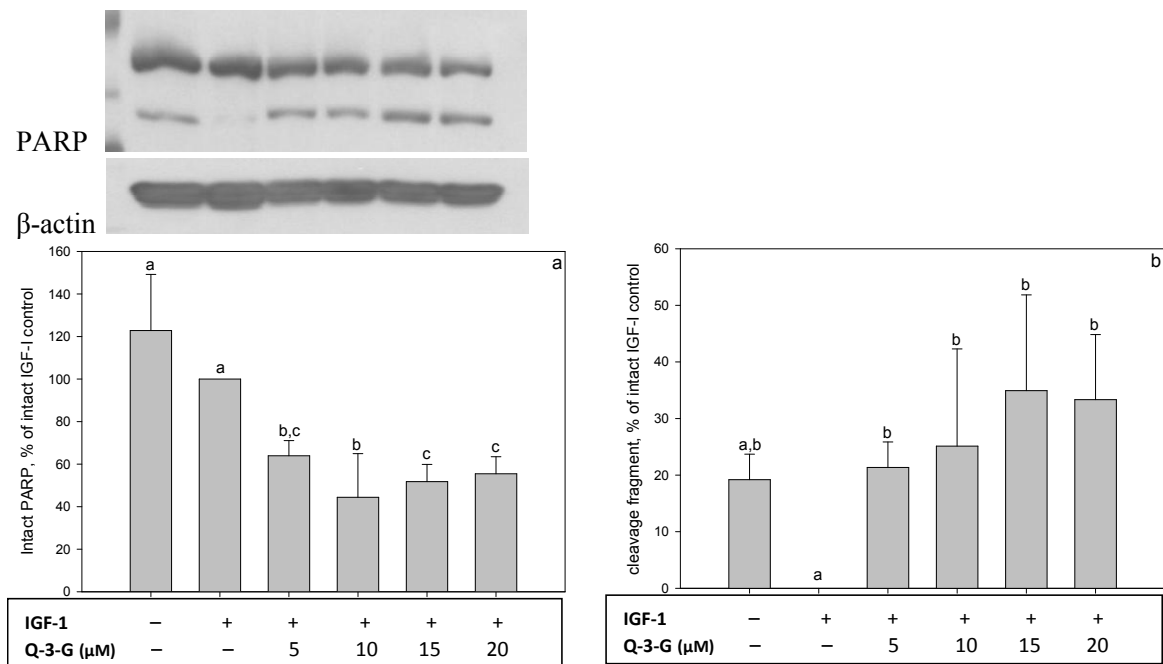
**Figure 3.12. Acridine Orange/Ethidium Bromide (AO/EB) Staining of MCF-7 cells treated with Q-3-G.** MCF-7 cells were serum-starved for 12 hours, then treated for 24 hrs with varying doses of Q-3-G. (All treatments contained 100 ng/mL IGF-I.) Cells were harvested by scraping followed by centrifugation, then resuspended in AO/EB solution on ice in the dark. After 2 minutes, 10  $\mu$ L of sample were placed on a slide for counting under fluorescence microscopy using a 488 nm illumination/520 nm emission filter. Cells with uniform green appearance were scored as healthy, cells with bright condensed yellow or orange nuclei as apoptotic, and dark red cells as dead (a). Addition of Q-3-G to the IGF-I increased the proportion of apoptotic cells in a dose-dependent manner (b), while the background number of dead cells did not differ significantly among treatments (c). (Mean  $\pm$  SD, n = 3. Bars with no letters in common are statistically significantly different,  $P < 0.05$ .)

PARP Cleavage. Figure 3.12 shows the effects of Q-3-G on PARP cleavage in MCF-7 cells. The upper band represents the intact PARP protein, the expression of which was statistically the same between the SFM and IGF-I controls, and which decreased upon treatment with Q-3-G (Figure 3.14a). The expression of the PARP cleavage fragment, which indicates apoptosis, is presented as percent of the expression



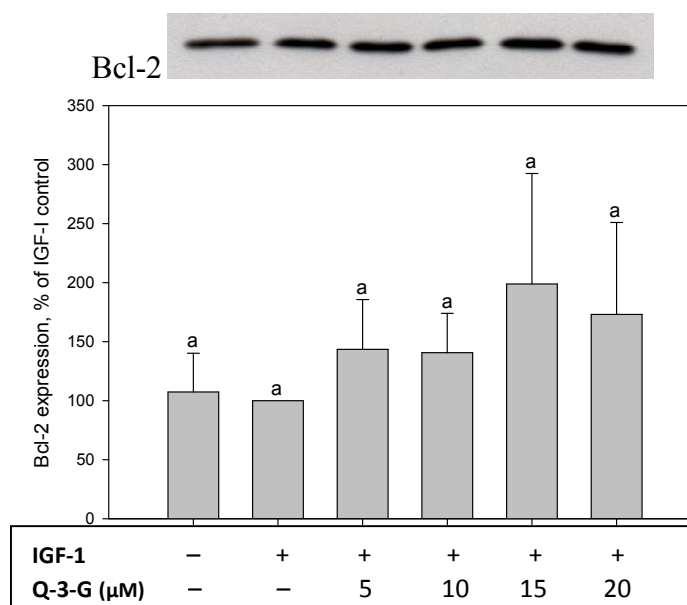
**Figure 3.13. Effect of Q-3-G on Caspase-9 Cleavage.** The figure represents expression of the cleavage fragment of Caspase-9 (lower band), as a percent of the IGF-I control (the upper band is the intact Caspase-9 protein). No significant difference existed between the SFM and IGF-I controls. However, the administration of Q-3-G in addition to the IGF-I increased Caspase-9 cleavage to  $221.8 \pm 65.5\%$ ,  $234.2 \pm 57.8\%$ ,  $248.1 \pm 131.8\%$ , and  $221.3 \pm 62.4\%$  of the IGF-I control in doses of 5, 10, 15 and 20  $\mu\text{M}$ , respectively. (Mean  $\pm$  SD,  $n=3$ . Bars with no letters in common are statistically significantly different,  $P < 0.05$ .)

of the intact PARP protein in the IGF-I control treatment (Figure 3.14b). Upon addition of Q-3-G, the corresponding increase of the 89 kDa fragment of PARP in a dose-dependent manner indicated that PARP cleavage and therefore apoptosis had occurred. Note the complete absense of the 89 kDa fragment in the positive control, where IGF-I suppressed PARP cleavage. Addition of only 5  $\mu\text{M}$  Q-3-G reversed this effect, returning the level of cleavage fragment to  $21.4 \pm 45\%$  of the IGF-I intact control, a level similar to that of the SFM control ( $19.2 \pm 4.5\%$ ). Doses of 10, 15 and 20  $\mu\text{M}$  Q-3-G induced PARP cleavage to  $25.1 \pm 17.2$ ,  $34.9 \pm 16.9$ , and  $33.3 \pm 11.5\%$  of the intact IGF-I control, respectively (mean  $\pm$  S.D.,  $n = 3$ ).



**Figure 3.14. Effect of Q-3-G on PARP Cleavage.** After 24 hours treatment, 5, 10, 15 and 20 μM Q-3-G decreased expression of intact PARP (upper band) to  $63.91 \pm 7.15\%$ ,  $44.40 \pm 20.55\%$ ,  $51.78 \pm 8.07\%$ , and  $55.47 \pm 8.02\%$  of the intact PARP expression in the IGF-I control, respectively (a). Upon addition of Q-3-G, the corresponding increase of the 89 kDa fragment of PARP (lower band) in a dose-dependent manner indicated that apoptosis and therefore PARP cleavage had occurred (b). Note the complete absence of the 89 kDa fragment in the IGF-I control, where IGF-I suppressed PARP cleavage. Addition of only 5 μM Q-3-G reversed this effect, returning the level of cleavage fragment to  $21.4 \pm 4.5\%$  of the intact PARP expression in the IGF-I control, a level similar to that of the SFM control ( $19.2 \pm 4.5\%$ ). Doses of 10, 15 and 20 μM Q-3-G induced PARP cleavage to  $25.1 \pm 17.2\%$ ,  $34.9 \pm 16.9\%$ , and  $33.3 \pm 11.5\%$  of the intact IGF-I control, respectively. (Mean  $\pm$  SD, n = 3. Bars with no letters in common are statistically significantly different,  $P < 0.05$ .)

**Bcl-2.** Doses of 5, 10, 15 and 20 μM Q-3-G increased Bcl-2 expression relative to the IGF-I control (Figure 3.15). However, none of these differences was statistically significant (mean  $\pm$  S.D., n=3).



**Figure 3.15. Effect of Q-3-G on Bcl-2 Expression.** Membranes from Cycin D1 (Figure 3.6) were reprobed for Bcl-2 expression. 5, 10, 15 and 20 μM Q-3-G increased Bcl-2 expression relative to the IGF-I control. However, none of these differences was statistically significant. (Mean ± SD, n=3. Bars with no letters in common are statistically significantly different,  $P < 0.05$ .)

## DISCUSSION

Insulin-like Growth Factor I (IGF-I) is a growth factor produced by the liver for endocrine functions and by many other tissues for autocrine and paracrine functions (Yakar *et al.*, 1999). IGF-I acts through binding to the IGF-IR. The IGF-IR is 70% homologous to the insulin receptor, but has independent roles in cell proliferation and survival, differentiation, and neoplastic growth (Baserga *et al.*, 2003). As such, the IGF-IR has become an attractive target for cancer research.

Nutrition, the IGF-I system, and cancer risk are all closely intertwined. The three major factors influencing endocrine IGF-I production in the liver are growth hormone (Bichell *et al.*, 1992), insulin (Kaytor *et al.*, 2001; Kaytor *et al.*, 2001b) and



nutritional status (caloric and protein intakes) (Clemmons *et al.*, 1981; Thissen *et al.*, 1994). Caloric restriction studies in a p53-deficient mouse bladder cancer model, which reduced cancer incidence, were also shown to reduce IGF-I levels (Dunn *et al.*, 1997). Adding IGF-I back to the circulation of the mice reversed the effect on cancer incidence. Higher levels of GH are associated with higher cancer risk (Maison *et al.*, 1998).

The results of this study have shown that Q-3-G reverses the mitogenic and anti-apoptotic effects of IGF-I in MCF-7 cells *in vitro*. Q-3-G inhibited MCF-7 cell proliferation more potently than quercetin, and Q-3-G inhibited IGF-I-stimulated cell proliferation much more potently than FBS-stimulated proliferation (Figure 3.3). Most of the remaining markers that were investigated showed the same pattern of IGF-I-mediated stimulation or down-regulation of a response compared to the SFM control cells, followed by reversal of that signal upon addition of the Q-3-G. Taken together, these data indicated that Q-3-G was clearly targeting IGF-IR signaling.

According to the cell cycle analysis (Figure 3.5), IGF-I stimulated cell proliferation by promoting cell cycle progression as expected. After 24 hours, IGF-I-treated cells had a smaller proportion of the population in G0/G1 phase, and a greater proportion in S and G2/M phases, compared to the SFM control (Figure 3.5a-c). Treatment with Q-3-G along with the IGF-I reversed this effect, returning the cell cycle distribution to that similar to the SFM control. The corresponding sub-G0 fragment data (Figure 3.11a) show that in these 24 hour samples, 6.4% of the events were sub-G0 fragments in the SFM control, 3.4% were sub-G0 in the IGF-I control, and 4.6% were sub-G0 after treatment with 15  $\mu$ M Q-3-G in addition to the 100 ng/mL IGF-I. Therefore, ~95% of the events in the samples were intact nuclei. Of these populations of intact nuclei, IGF-I decreased the percentage of G0/G1 cells from

90.1% in the SFM control to 78.2% in the IGF-I control, indicating progression past the G1/S checkpoint. 15  $\mu$ M of Q-3-G reversed the effects of IGF-I, increasing the proportion of cells in the G0/G1 phase to 87.2%. After 48 hours treatment, though the proportions were different, the patterns looked the same. 9.5% of the singlet events in the SFM control and 4.8% in the IGF-I control were sub-G0 fragments (Figure 3.11b). Treatment with 15  $\mu$ M Q-3-G dramatically increased this to 44.9%. Of the remaining singlet nuclei in the samples, 91.0% and 82.0% of the SFM and IGF-I controls, respectively, were in G0/G1 phase (Figure 3.5d). Addition of 15  $\mu$ M Q-3-G to the IGF-I increased this to 88.5%, showing the same reversal of the effects of IGF-I seen after 24 hours. Since the sub-G0 peak in the 48 hour samples of doses 3 through 15  $\mu$ M Q-3-G approached the origin of the histogram, the proportion of sub-G0 fragments may in fact be an underestimate. Therefore, the sub-G0 peak may be used only as an indication that cell death is occurring in the samples in a time- and dose-dependent manner, but cannot be used as an absolute quantification of apoptosis.

Additional apoptosis markers showed patterns similar to the flow cytometry results. IGF-I suppressed apoptosis compared to SFM control as demonstrated by PARP cleavage (an early event; Figure 3.14), AO/EB staining (showing early and late apoptotic cells; Figure 3.12), and the sub-G0 peak (a late event, Figure 3.11) as mentioned above. Treatment with Q-3-G concomitantly with the IGF-I reversed these effects. Therefore, Q-3-G has blocked both of the IGF-IR's main cell survival mechanisms: driving cell cycle progression and suppressing apoptosis.

The one apoptosis marker which did not respond significantly to treatment was Bcl-2 (Figure 3.15). Expression of Bcl-2 in the IGF-I control was slightly lower than the SFM control, while Q-3-G appeared to increase expression over both the SFM and IGF-I controls. Since Bcl-2 is a pro-survival protein (Adams and Cory, 1998), this

pattern is inconsistent with results of other apoptotic markers in this study, where Q-3-G induced apoptosis. On the other hand, Bcl-2 is known to have cell cycle-modulatory functions that involve sections of the Bcl-2 protein which are unrelated to its anti-apoptotic functions. In response to inadequate growth conditions, Bcl-2 prompts entry into quiescence and delays return to the cell cycle (Adams and Cory, 1998, and sources therein). This function is consistent with the results shown here: after 24 hours treatment, Q-3-G induces G1 cell cycle arrest and a corresponding up-regulation of Bcl-2. Nevertheless, the differences in Bcl-2 expression were not statistically significant between any of the treatment groups and therefore this cannot be regarded as a major mechanism of action of the Q-3-G. The lack of significance may be due to the large variation between gels, and/or to sub-optimal western blotting or imaging conditions for this particular protein. Further investigation would be needed to address these uncertainties.

IGF-I Receptor expression in MCF-7 cells increased with Q-3-G treatment. In the absence of IGF-I, IGF-IR phosphorylation was completely absent, verifying the model. After 12 and 24 hours of treatment, the pattern of IGF-IR phosphorylation mirrored that of the receptor expression, indicating that any effect of treatment on the receptor was on expression and not on phosphorylation (Figure 3.6). Therefore, any down-stream effects seen, such as the down-regulation of Akt phosphorylation (Figure 3.7b) and Cyclin D1 expression (Figure 3.8), were clearly not due to an inhibition of IGF-IR activation. Increased expression of IGF-IR has been seen in cancer cell lines and tumors, and is associated with aggressive tumor phenotypes in some types of cancer such as synovial sarcoma (Xie *et al.*, 1999) and lung carcinoma (Long *et al.*, 1998). In breast tumors, however, while IGF-IR expression was elevated compared to

normal tissue, this up-regulation was associated with a more favorable prognosis (Papa *et al.*, 1993).

The ratio of IGF-IR to IRS-1 expression is an important factor determining whether the receptor activates mitogenic or differentiation pathways. A high IGF-IR to IRS-1 ratio favors differentiation, which is a cancer-protective effect (Valentinis and Baserga, 2001). In a rat prostate cancer cell line, quercetin altered this ratio by reducing IRS-1 as well as inhibiting the IGF-I-induced up-regulation of the receptor (Wang *et al.*, 2003). Genistein has been shown to affect differentiation markers in MCF-7 (Constantinou *et al.*, 1998). After 12 and 24 hours of treatment, Q-3-G in addition to IGF-I treatment caused a clear up-regulation of IGF-IR in this study. This may help to drive the cells toward differentiation. At least for the doses and treatment times employed in this study, the increased IGF-IR expression occurred concomitantly with significantly reduced Akt activation. Therefore it is believed the increased IGF-IR expression may be functioning much like that induced by the quercetin mentioned above in the rat prostate cancer cells, which promoted cellular differentiation rather than mitogenesis. Future studies may investigate the effects of Q-3-G on the IRS-1 expression in MCF-7 cells, as well as the IGF-IR-mediated differentiation pathways in MCF-7 cells.

In this study, Akt phosphorylation was clearly induced by IGF-I treatment, and abrogated upon addition of Q-3-G to the IGF-I (Figure 3.7b). Across all treatments, total Akt expression did not change. As the Q-3-G did not inhibit IGF-IR phosphorylation, the point of action is presumably downstream of the receptor and upstream of Akt. The most likely target is PI3K, as quercetin aglycone has been demonstrated to be a PI3K inhibitor (Matter *et al.*, 1992; Agullo *et al.*, 1997). Akt phosphorylation at the Ser<sup>473</sup> residue was investigated in this study. In order to be fully

activated, Akt must be phosphorylated on two key residues: Ser<sup>473</sup> and Thr<sup>308</sup>. Thr<sup>308</sup>, which occurs in the activation loop of the protein, is phosphorylated by PDK-1 (Alessi *et al.*, 1996). mTOR phosphorylates Ser<sup>473</sup> in the carboxy terminus (Sarbasov *et al.*, 2005). Phosphorylation of both of these residues is required for full Akt activation, both are stimulated by IGF-I treatment, and phosphorylation of one is not dependent on the phosphorylation state of the other (Alessi *et al.*, 1996). The pattern of the bands on the Western Blots show one visible band in the SFM control, and two visible bands at all other treatments, presumably from an electrophoretic mobility shift. Since the upper band, presumably the Akt with a higher level of phosphorylation, is the band that diminishes upon Q-3-G treatment, the authors speculate phosphorylation of a residue other than Ser<sup>473</sup> was being diminished. In this study, Cyclin D1 was clearly up-regulated by IGF-I and clearly down-regulated by concomitant treatment with Q-3-G and IGF-I (Figure 3.8). Since Akt phosphorylation was significantly down-regulated by the Q-3-G treatment in this study, it is possible that Cyclin D1 expression was down-regulated by interference with both of Akt's actions: driving Cyclin D1 transcription, and suppressing Cyclin D1 degradation.

As summarized by Clark *et al.* (2005), p21 was originally known as a Cyclin-dependent Kinase Inhibitor (CKI) and could induce cell cycle arrest when triggered by p53 in an ERK- and p38-dependent fashion. In MCF-7 cells treated with IGF-I, however, p21 aided in cell proliferation induced by the IGF-I in an ERK-dependent manner. Additionally, the roles of IGF-I in Cyclin D1 up-regulation and Rb phosphorylation were determined to be PI3K-dependent and ERK-independent in MCF-7 cells and these functions did not depend on p21. In a study by Clark *et al.* investigating the role of IGF-I in DNA repair, in acute assays (1 through 6 hours) the IGF-I-induced expression of p21 was dependent on both PI3K and ERK pathways.

Chronic (24 and 48 hours) induction of p21 expression by IGF-I was found to be partially dependent on PI3K activity and was associated with increased DNA synthesis. The authors conclude that in MCF-7 cells suffering DNA damage as a result of bleomycin treatment, IGF-I induced p21, which aided in cell cycle arrest (Clark *et al.*, 2005). In the current study, although not statistically significant, the pattern of p21 expression (Figure 3.10a) is consistent with what is known regarding the functions of p21 in response to IGF-I and the pathways involved. All samples treated with IGF-I showed p21 expression somewhat higher than the SFM control. However, this induction was not affected by addition of Q-3-G. IGF-I may have induced p21 expression via an ERK-dependent pathway to induce cell proliferation. Q-3-G then inhibited cell cycle and apoptosis via a PI3K pathway, thus inhibiting proliferation without altering p21 expression.

IGF-I down-regulates p27 by increasing the levels of Skp2, a ubiquitin ligase for p27, thereby stimulating ubiquitination and subsequent proteasome degradation of p27. This process is believed to involve the PI3K pathway (Yuhong *et al.*, 2003). The results of the current study show a slight decrease in p27 expression after treatment with IGF-I compared to the SFM control (Figure 3.10b). Unexpectedly, addition of Q-3-G to the IGF-I treatment further down-regulated p27 expression. While a trend is visible, the lack of statistical significance and large variation render these data inconclusive.

Proliferating cell nuclear antigen (PCNA), often used as a marker of cell proliferation, is a DNA clamp which serves to increase the processivity of DNA polymerase during DNA replication and repair and is under the transcriptional control of E2F transcription factors (Krishna *et al.*, 1994; Kelman and O'Donnell, 1995; Tommasi and Pfeifer, 1999; Ahn *et al.*, 2002; Maga and Hubescher, 2003). As such,

one would expect to see an increase in PCNA expression upon addition of IGF-I to the serum-starved MCF-7 cells in this study, and a decrease in expression upon addition of the Q-3-G, when reduced cell proliferation and cell cycle arrest are evident. This pattern is seen (Figure 3.10c) although the differences between treatment groups are slight and non-significant. This could be attributed to the timing of the experiment. Both the Cyclin D1 and PCNA expression were measured after 24 hours of treatment. However, since PCNA is controlled by E2F transcription factors, and Cyclin D1/CDK-4 regulate E2F activity by phosphorylating Rb, PCNA expression may occur slightly later. Perhaps after a longer treatment period larger differences in PCNA expression may become apparent.

The antioxidant properties of phytochemicals have gained much attention in recent years, since oxidative stress has been linked with chronic diseases such as cancer, and reactive oxygen species (ROS) can function as signaling molecules within cells. Lin *et al.* (2007) demonstrated that IGF-I and estrogen enhance one another's cell proliferative activity in MCF-7 cells, through a ROS-dependent pathway which results in phosphorylation of IRS-1, ERKs and JNKs. The aglycones quercetin and baicalein inhibited this effect, while their respective glycosides, quercitrin (quercetin-3-*O*-rhamnoside), rutin (quercetin-3-*O*-rutinoside), and baicalin (baicalein-7-*O*-glucuronide), did not. Plumb *et al.* (1999) found that quercetin aglycone had more potent antioxidant activity than two glycosides (quercetin 3-*O*-glucuronide and quercetin 3-*O*-rutinoside) using the Trolox equivalent antioxidant capacity (TEAC) assay. The cellular antioxidant activity (CAA) assay results seen in the current study are consistent: quercetin had potent antioxidant activity in MCF-7 cells with an EC<sub>50</sub> of  $6.28 \pm 0.25 \mu\text{M}$ , while Q-3-G was a much weaker antioxidant activity with an EC<sub>50</sub> of  $106.63 \pm 13.26 \mu\text{M}$  (Figure 3.4 and Table 3.1). The doses of Q-3-G used for the remainder of experiments in this study ranged from 1 to 20  $\mu\text{M}$ . In the CAA assay,

20  $\mu$ M of Q-3-G showed little to no antioxidant activity. Therefore, we conclude that the effects of Q-3-G on IGF-I-induced proliferation and signal transduction seen in this study cannot be attributed to quenching of ROS.

ER $\alpha$  functions via genomic events in the nucleus, and via signal transduction events in the plasma membrane (Mawson, *et al.* 2005). The ER and IGF-IR pathways collaborate to promote cell cycle progression in various ways. In the nucleus, estrogen has been shown to increase the transcription of IGF-I, IGF-IR, and IRS-1. IGF-I-treated ER<sup>+</sup> MCF-7 cells demonstrated increased ER transcriptional activity. Estrogen increases expression of c-myc, which blocks p21, while insulin increases expression of Cyclin E. Estrogen, insulin, and IGF-I also promote expression of Cyclin D1. Together these events lead to enhanced progression through the G1-S transition. At the plasma membrane, estrogen-bound ER may bind and activate the IGF-IR or bind the p85 subunit of PI3K and Src, directly activating the PI3K and p21/ras/MAPK pathways (Mawson, *et al.* 2005).

ER can be activated by phosphorylation induced by estrogen or other growth factors such as EGF and IGF-I (Kato *et al.*, 1995; Bunone *et al.*, 1996; Fagan and Yee 2008). Activation function domain-1 (AF-1) of the ER is regulated by growth factors, while AF-2 is activated by estrogen. IGF-I can induce ER activation in two ways: phosphorylation of Ser<sup>118</sup> of the AF-1 by MAPK, and phosphorylation of Ser<sup>167</sup> of AF-1 by Akt (Ignar-Trowbridge *et al.*, 1993; El-Tanani and Green, 1997).

In both normal and cancerous breast tissue, IGF-I and estrogen both work to potentiate one another's transcriptional activity (Fagan and Yee, 2008). In the absence of estrogen, treatment with IGF-I increased transcriptional activity of the ER in MCF-7L cells (Lee *et al.*, 1997). IGFBP-1 inhibited ER activation by both IGF-I and



by estrogen (Figuerola *et al.*, 1993). The implication is that IGF-I is necessary for the ER to reach its full activation potential stimulated by estrogen (Fagan and Yee, 2008).

Cascio *et al.* (2007) provide further evidence of the crosstalk between IGF-I and ER signaling in MCF-7 cells. While IGF-I (50 nM) can stimulate nuclear localization of the ER, it is less efficient than E2 (10 nM). After 1 and 4 hrs of treatment, IGF-I stimulated transcription of Cyclin D1 mRNA more efficiently than E2 or both in combination. IGF-I recruited ER to the Cyclin D1 AP-1 sequence to fully activate transcription of Cyclin D1, although IGF-I alone recruited less ER than E2 had recruited. However, even in the absence of the ER (knockdown by RNA interference), IGF-I was able to stimulate some Cyclin D1 transcription (Cascio *et al.*, 2007).

In the current study, it is quite possible the IGF-I treatment stimulated nuclear translocation of ER and recruitment of ER and its co-regulators to the AP-1 site to promote Cyclin D1 transcription. However, since all treatments are devoid of E2, then these actions of ER cannot be expected to reach full potential. As mentioned earlier, IGF-I can activate ER via phosphorylation of Ser<sup>167</sup> of the AF-1 site by Akt. Treatment with Q-3-G clearly down-regulated phosphorylated Akt. Therefore, a subsequent reduction in phosphorylation of ER at Ser<sup>167</sup> would be expected. The crosstalk that occurs between IGF-IR and ER signaling must always be kept in mind. IGF-I-stimulated growth is inhibited by anti-estrogens, and estrogen-stimulated growth is inhibited by anti-IGF-I treatments (Wakeling *et al.*, 1989; Freiss *et al.*, 1990). IGF-I signaling may contribute to resistance against anti-estrogen treatments in breast cancers. Therefore, a compound that targets IGF-IR signaling in breast cancer may be especially valuable.

Q-3-G was much more potent than the quercetin aglycone in inhibiting IGF-I-mediated MCF-7 cell proliferation. This may be attributed to greater bioavailability of

the glycoside compared to quercetin aglycone (Hollman and Katan, 1998). The following studies investigated the influence of IGF-I on cellular glucose transporters, which play a significant role in the absorption of quercetin glycosides (Lostao *et al.*, 1994; Gee *et al.*, 1998; Kellett, 2001).

Neonatal rats, ages 8-12 days, were fed rat milk substitute (RMS) which contained no growth factors, or RMS supplemented with IGF-I or IGF-II in concentrations which approximated those found in rat milk. Control rats were naturally dam-fed (Lane *et al.*, 2002). While serum glucose levels in the RMS-fed rat pups were lower than dam-fed pups, addition of IGF-I or IGF-II returned blood glucose to normal levels. The same pattern was seen in SGLT1 mRNA expression in the mid-jejunum: RMS-fed pups had lower mRNA levels than dam-fed, while mRNA levels in pups supplemented with IGF-I or IGF-II were similar to the dam-fed controls. Histological localization of the SGLT1 transporter corroborated the mRNA results: no positive cells were visible in samples from the RMS group, while the IGF-I and IGF-II supplemented groups showed similar staining to the dam-fed group. Expression of GLUT2 mRNA in the mid-jejunum of RMS-fed pups was also lower than the dam-fed controls. Levels in IGF-I-supplemented pups were not statistically significantly different from the RMS group, while levels in the IGF-II-supplemented pups were not statistically significantly different from the dam-fed controls (Lane *et al.*, 2002).

The jejunal uptake of glucose in neonatal piglets fed an IGF-I-supplemented RMS was reported (Alexander and Carey, 1999 and 2001). The researchers found the IGF-I increased glucose uptake, via PI3K activation, but saw no change in SGLT1 expression. Lane *et al.* speculate this may be because Alexander and Carey used piglets ages 1-5 days, a time when SGLT1 expression may be below detection limits

of Western Blotting, as the expression of SGLT1 and GLUT2 increase during the suckling period of the neonates. Also, Alexander and Carey used a porcine RMS containing oxytetracycline which inhibits mitochondrial protein synthesis, and neomycin which may affect protein synthesis and processing. Inclusion of these antibiotics may also explain the lack of difference in SGLT1 expression seen in this model (Lane *et al.*, 2002). Lane concludes the difference in glucose uptake in the piglet model due to IGF-I supplementation may have been the result of SGLT1 translocation, rather than changes in expression (Lane *et al.*, 2002). In their own rat pup model, Lane *et al.* summarize that IGF-I and IGF-II play a role in the developing jejunum's ability to absorb glucose from the intestinal lumen.

In light of the known functions of IGF-I in fetal and early childhood growth, and the fact that insulin stimulates IGF-I production, it makes sense that IGF-I would be involved in glucose transport into the cell, particularly in the small intestine. Intake of food triggers insulin which stimulates IGF-I production. IGF-I may stimulate growth and other metabolic functions by up-regulating expression of glucose transporters so that cells may absorb the available glucose.

In MCF-7 cells, SGLT1 is expressed in very low levels, if expressed at all. For example, in a study investigating the tyrosine kinase-independent functions of EGFR in cancer cell survival, SGLT1 mRNA was detected by RT-PCR, but the corresponding protein was not detectable by western blotting (Weihua *et al.*, 2008). However, another group found a novel glucose transporter, GLUT12, to be expressed in MCF-7 cells (Rogers *et al.*, 2002). GLUT12 is normally only expressed in skeletal muscle, heart, and fat, all insulin-sensitive tissues. In MCF-7 cells grown without insulin, the GLUT12 was localized to the perinuclear area, and continuous growth in insulin showed GLUT12 expression in the membrane. However, they were not able to

show an acute re-localization response to insulin (Rogers *et al.*, 2002). Later the same group found GLUT12 to be expressed in breast cancer tumors but not in the corresponding healthy mammary tissues from the same patients (Rogers *et al.*, 2003).

Similarly, another group found the GLUT-1 transporter to be expressed in all 12 primary breast tumors and all 8 lymph node metastases tested (Brown and Wahl, 1993). In normal mammary tissue from the same patient, the GLUT-1 expression was lower than that seen in the respective tumor. They suggested this may partly explain the selectivity of 2-[18F]-fluoro-2-deoxy-D-glucose (FDG) for tumor cells, which is used as a marker for increased glucose metabolism enabling visualization of tumors by positron emission tomography (PET) imaging. In 2008, Ong *et al.* (2008) demonstrated that higher expression of GLUT-1 and hexokinase II, a glycolytic enzyme, correlated with the uptake of FDG in 5 human cancer cell lines (AGS, A431, A549, Colo 320 HSR, and HepG2). Fibroblast control cells had lower expression of GLUT-1 and hexokinase II than did the cancer cell lines (Ong *et al.*, 2008). In general, tumor cells are known to have higher glucose demand (Weber, 1977) and higher expression of glucose transporters (Smith, 1999) than normal cells.

Whether SGLT1, GLUT 12, or another transporter, the possibility exists in our model that IGF-I treatment may cause up-regulation or translocation of one or more glucose transporters in the MCF-7 cells, thereby allowing a route of increased absorption of Q-3-G into the cells. This connection may explain why the Q-3-G was so much more dramatically effective in the IGF-I-treated cells than the FBS-treated cells, and why Q-3-G was so much more effective than the quercetin aglycone, which may only enter the cells through passive diffusion. This hypothesis warrants further study, as mechanisms which improve delivery of an anti-cancer agent to a cancer cell are valuable. Walgren *et al.* (2000) warns caution in such lines of thinking regarding

drug design: though a glucose moiety may facilitate transport of a chemical into a cell, it also facilitates efflux out of the cell via the multidrug resistance protein-2 (MRP-2). MCF-7 cells do not express MRP-1 (Zhang and Morris, 2003), MRP-2 (Smitherman *et al.*, 2004), or P-glycoprotein (Fairchild *et al.*, 1990; Zhang and Morris, 2003). The absence of all three of these efflux transporters may contribute to the efficacy of Q-3-G in the current study.

This study shows Q-3-G to be a very potent, very effective inhibitor of IGF-I signaling in MCF-7 cells *in vitro*. Future studies will add insight into the mechanisms of action. Such studies may include an assessment of: the IGF-IR to IRS-1 ratio to determine whether the increased receptor expression may drive cells into a differentiation pathway; IRS-1 expression and phosphorylation; PI3K expression, activation, and kinase activity; Akt phosphorylation at other key residues; and effects on AP-1 and GSK-3 in Cyclin D1 transcription and degradation. The dramatic potency of Q-3-G compared to quercetin is intriguing. Thus, future studies should include assessment of the effects of IGF-I on expression and localization of glucose transporters, and mechanisms of absorption, metabolism, and efflux of Q-3-G in MCF-7 cells as well as other normal and cancerous mammary cell lines. The IGF-I system is a critical component of breast cancer signaling, and the connections between diet and cancer are clear. The results of this study help to explain the mechanisms by which apple consumption may reduce breast cancer risk. Additionally, such studies of purified compounds contribute to the understanding of cancer cell behavior and may inform future endeavors in chemotherapeutic drug design.

## REFERENCES

- Adams, J.M. and S. Cory. 1998. The Bcl-2 protein family: arbiters of cell survival. *Science*. 281(5381): 1322-1326.
- Agullo, G., L. Gamet-Payrastre, S. Manenti, C. Viala, C. Remesy, H. Chap and B. Payrastre. 1997. Relationship between flavonoid structure and inhibition of phosphatidylinositol 3-kinase: a comparison with tyrosine kinase and protein kinase C inhibition. *Biochemical Pharmacology*. 53: 1649-1657.
- Ahn, J.D., R. Morishita, Y. Kaneda, H-S. Kim and I-K. Lee. 2002. Novel E2F decoy oligodeoxynucleotides inhibit in vitro vascular smooth muscle cell proliferation and in vivo neointimal hyperplasia. *Gene Therapy*. 9(24): 1682-1692.
- Alessi, D.R., M. Andjelkovic, B. Caudwell, P. Cron, N. Morrice, P. Cohen, and B.A. Hemmings. 1996. Mechanism of activation of protein kinase B by insulin and IGF-I. *EMBO Journal*. 15(23): 6541-6551.
- Alexander, A.N. and H.V. Carey. 1999. Oral IGF-I enhances nutrient and electrolyte absorption in neonatal piglet intestine. *AJP-Gastrointestinal and Liver Physiology*. 277: G619-G625.
- Alexander, A.N. and H.V. Carey. 2001. Involvement of PI 3-kinase in IGF-I stimulation of jejunal Na<sup>+</sup> -K<sup>+</sup> - ATPase activity and nutrient absorption. *AJP-Gastrointestinal and Liver Physiology*. 280: G222-G228.
- American Cancer Society. 2008. Cancer Facts & Figures 2008. American Cancer Society, Atlanta.
- Baserga, R., F. Peruzzi and K. Reiss. 2003. Mini Review: The IGF-I Receptor in Cancer Biology. *International Journal of Cancer*. 107: 873-877.
- Bichell, D.P., K. Kikuchi and P. Rotwein. 1992. Growth hormone rapidly activates insulin-like growth factor I gene transcription in vivo. *Molecular Endocrinology*. 6: 1899-1908.

- Block, G., B. Patterson and A. Subar. 1992. Fruit, vegetables and cancer prevention: a review of the epidemiological evidence. *Nutrition and Cancer*. 18(1): 1-29.
- Boyer, J. and R.H. Liu. 2004. Apple phytochemicals and their health benefits. *Nutrition Journal*. 3(5).
- Brown, R.S. and R.L. Wahl. 1993. Overexpression of Glut-1 glucose transporter in human breast cancer. An immunohistochemical study. *Cancer*. 72(10): 2979-2985.
- Bunone, G., P.A. Briand, R.J. Miksicek and D. Picard. 1996. Activation of the unliganded estrogen receptor by EGF involves the MAP kinase pathway and direct phosphorylation. *EMBO*. 15: 2174-2183.
- Byrne, C., G.A. Colditz, W.C. Willett, F.E. Speizer, M. Pollak and S.E. Hankinson. 2000. Plasma insulin-like growth factor (IGF) I, IGF-binding protein 3, and mammographic density. *Cancer Research*. 60: 3744-3748.
- Cantley, L.C. 2002. The Phosphoinositide 3-kinase Pathway. *Science*. 296: 1655-1657.
- Cardone, M.H., N. Roy, H.R. Stennicke, G.S. Salvesen, T.F. Franke, E. Stanbridge, S. Grisch and J.C. Reed. 1998. Regulation of cell death protease caspase-9 by phosphorylation. *Science*. 282: 1318-1321.
- Cascio, S., V. Bartella, C. Garofalo, A. Russo, A. Giordano and E. Surmacz. 2007. Insulin-like growth factor 1 differentially regulates estrogen receptor-dependent transcription at estrogen response element and AP-1 sites in breast cancer cells. *The Journal of Biological Chemistry*. 282: 3498-3506.
- Chan, J.M., M.J. Stampfer, E. Giovannucci, P.H. Gann, J. Ma, P. Wilkinson, C.H. Hennekens and M. Pollak. 1998. Plasma insulin-like growth factor-I and prostate cancer risk: A prospective study. *Science*. 279: 563-566.
- Chang, H.C., M.I. Churchwell, K.B. Delclos, R.R. Newbold and D.R. Doerge. 2000. Mass spectrometric determination of genistein tissue distribution in diet-exposed Sprague-Dawley rats. *Journal of Nutrition*. 130: 1963-1970.

- Clark, M.A., C.M. Perks, Z.E. Winters and J.M.P. Holly. 2005. DNA damage uncouples the mitogenic response to IGF-I in MCF-7 malignant breast cancer cells by switching the roles of PI3 kinase and p21(WAF1/Cip1). *International Journal of Cancer*. 116(4): 506-513.
- Clemmons, D.R., A. Klibanski, L.E. Underwood, J.W. McArthur, E.C. Ridgway, I.Z. Beitins, et al. 1981. Reduction of plasma immunoreactive somatomedin-C during fasting in humans. *Journal of Clinical Endocrinology and Metabolism*. 53: 1247-1250.
- Constantinou, A.I., A.E. Krygier and R.R. Mehta. 1998. Genistein induces maturation of cultured human breast cancer cells and prevents tumor growth in nude mice. *American Journal of Clinical Nutrition*. 68: 1426S-1430S.
- Cotter, T.G. and T.G. Martin. 1996. Techniques in Apoptosis. A User's Guide. Portland Press Ltd., London.
- Downward, J. 1999. How Bad phosphorylation is good for survival. *Nature Cell Biology*. 1: E33-35.
- Dufourny, B., J. Alblas, H.A.A.M. van Teeffelen, F.M.A. van Schaik, B. vander Burg, P.H. Steenbergh and J.S. Sussenbach. 1997. Mitogenic signaling of insulin-like growth factor I in MCF-7 human breast cancer cells requires phosphatidylinositol 3-kinase and is independent of mitogen-activated protein kinase. *The Journal of Biological Chemistry*. 272: 31163-31171.
- Dunn, S.E., F.W. Kari, J. French, et al. 1997. Dietary restriction reduces insulin-like growth factor I levels, which modulates apoptosis, cell proliferation, and tumor progression in p53-deficient mice. *Cancer Research*. 57: 4667-4672.
- El-Tanani, M.K. and C.D. Green. 1997. Two separate mechanisms for ligand-independent activation of the estrogen receptor. *Molecular Endocrinology*. 11: 928-937.
- Escarpa, A. and M. Gonzalez. 1998. High-performance liquid chromatography with diode-array detection for the performance of phenolic compounds in peel and pulp from different apple varieties. *Journal of Chromatography A*. 823: 331-337.



- Fagan, D.H. and D. Yee. 2008. Crosstalk between IGF1R and estrogen receptor signaling in breast cancer. *Journal of Mammary Gland Biology and Neoplasia*. 13: 423-429.
- Fairchild, C.R., J.A. Moscow, E.E. O'Brien and K.H. Cowan. 1990. Multidrug resistance in cells transfected with human genes encoding a variant P-glycoprotein and glutathione S-transferase-pi. *Molecular Pharmacology*. 37: 801-809.
- Felice, D.L., J. Sun and R.H. Liu. 2009. A modified methylene blue assay for accurate cell counting. *Journal of Functional Foods*. 1: 109-118.
- Figuroa, J.A., J. Sharma, J.G. Jackson, M.J. McDermott, S.G. Hilsenbeck and D. Yee. 1993. Recombinant insulin-like growth factor binding protein-1 inhibits IGF-I, serum, and estrogen-dependent growth of MCF-7 human breast cancer cells. *Journal of Cellular Physiology*. 157: 229-236.
- Freiss, G., H. Rochefort and F. Vignon. 1990. Mechanisms of 4-hydroxytamoxifen anti-growth factor activity in breast cancer cells: alterations of growth factor receptor binding sites and tyrosine kinase activity. *Biochemical and Biophysical Research Communications*. 173: 919-926.
- Gallus, S., R. Talamini, A. Giacosa, M. Montella, V. Ramazzotti, S. Franceschi, E. Negro and C. LaVecchia. 2005. Does an apple a day keep the oncologist away? *Annals of Oncology*. 16: 1841-1844.
- Gee, J.M., M.S. DuPont, M.J.C. Rhodes and I.T. Johnson. 1998. Quercetin glucosides interact with the intestinal glucose transport pathway. *Free Radical Biology and Medicine*. 25: 19-25.
- Geier, A., C. Weiss, R. Barry, M. Haimsohn, R. Hemi, Z. Malik and A. Karadik. 1995. Multiple pathways are involved in protection of MCF-7 cells against death due to protein synthesis inhibition. *Journal of Cellular Physiology*. 163(3): 570-576.
- Hankinson, S.E., W.C. Willett, G.A. Colditz, D.J. Hunter, D.S. Michaud, B. Deroo, B. Rosner, F.E. Speizer and M. Pollak. 1998. Circulating concentrations of insulin-like growth factor-I and risk of breast cancer. *Lancet*. 351(9113): 1393-1396.

- Hertog, M.G., P.C. Hollman, M.B. Katan and D. Kromhout. 1993. Intake of potentially anticarcinogenic flavonoids and their determinants in adults in the Netherlands. *Nutrition and Cancer*. 20(1): 21-29.
- Hollman, P.C. and M.B. Katan. 1998. Bioavailability and health effects of dietary flavonols in man. *Archives of Toxicology Supplement*. 20: 237-248.
- Holly, J.M., D.J. Gunnell and G. Davey Smith. 1999. Growth hormone, IGF-I and cancer. Less intervention to avoid cancer? More intervention to prevent cancer? *The Journal of Endocrinology*. 162(3): 321-330.
- Ignar-Trowbridge, D.M., C.T. Teng, K.A. Ross, M.G. Parker, K.S. Korach and J.A. McLachlan. 1993. Peptide growth factors elicit estrogen receptor-dependent transcriptional activation of an estrogen-responsive element. *Molecular Endocrinology*. 7: 992-998.
- Izumi, T., M.F. White, T. Kadowaki, F. Takaku, Y. Akanuma and M. Kasuga. 1987. Insulin-like growth factor I rapidly stimulates tyrosine phosphorylation of a Mr 185,000 protein in intact cells. *Journal of Biological Chemistry*. 262: 1282-1287.
- Jemal, A., R. Siegel, E. Ward, Y. Hao, J. Xu, T. Murray and M.J. Thun. 2008. Cancer statistics, 2008. *CA: A Cancer Journal for Clinicians*. 58: 71-96.
- Juskevich, J.C. and C.G. Guyer. 1990. Bovine growth hormone: Human food safety evaluation. *Science*. 249: 874-884.
- Kato, S., H. Endoh, Y. Masuhiro, T. Kitamoto, S. Uchiyama, H. Sasaki, et al. 1995. Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. *Science*. 270: 1491-1494.
- Kaytor, E.N., J.L. Zhu, C.I. Pao and L.S. Phillips. 2001. Insulin-responsive nuclear proteins facilitate Ap1 interactions with the insulin-like growth factor-I gene. *Journal of Biological Chemistry*. 276: 36896-36901.
- Kaytor, E.N., J.L. Zhu, C.I. Pao and L.S. Phillips. 2001b. Physiological concentrations of insulin promote binding of nuclear proteins to the insulin-like growth factor I gene. *Endocrinology*. 142: 1041-1049.

- Kellett, G.L. 2001. The facilitated component of intestinal glucose absorption. *Journal of Physiology*. 531: 585-595.
- Kelman, Z. and M. O'Donnell. 1995. Structural and functional similarities of prokaryotic and eukaryotic DNA polymerase sliding clamps. *Nucleic Acids Research*. 23: 3613-3620.
- Krishna, T.S., X.P. Kong, S. Gary, P.M. Burgers and J. Kuriyan. 1994. Crystal structure of the eukaryotic DNA polymerase processivity factor PCNA. *Cell*. 79: 1233-1243.
- Lane, R.H., Lane, R.H., B. Dvorak, N.K. MacLennan, K. Dvorakova, M.D. Halpern, T.D. Pham and A.F. Philips. 2002. IGF alters jejunal glucose transporter expression and serum glucose levels in immature rats. *AJP-Regulatory, Integrative and Comparative Physiology*. 283(6): R1450-R1460.
- Lawlor, M.A. and D.R. Alessi. 2001. PKB/Akt: a key mediator of cell proliferation, survival and insulin responses? *Journal of Cell Science*. 114: 2903-2910.
- Lee, A.V., C.N. Weng, J.G. Jackson and D. Yee. 1997. Activation of estrogen receptor-mediated gene transcription by IGF-I in human breast cancer cells. *Journal of Endocrinology*. 152: 39-47.
- Lee, K., Y. Kim, D. Kim, H. Lee and C. Lee. 2003. Major phenolics in apple and their contribution to the total antioxidant capacity. *Journal of Agricultural and Food Chemistry*. 51: 6516-6520.
- Lin, C.W., L.Y. Yang, S.C. Shen and Y.C. Chen. 2007. IGF-I plus E2 induces proliferation via activation of ROS-dependent ERKs and JNKs in human breast carcinoma cells. *Journal of Cellular Physiology*. 212: 666-674.
- Liu, R.H. and J. Sun. 2003. Antiproliferative activity of apples is not due to phenolic-induced hydrogen peroxide formation. *Journal of Agricultural and Food Chemistry*. 51(6): 1718-1723.
- Liu, R.H., J.R. Liu and B. Chen. 2005. Apples prevent mammary tumors in rats. *Journal of Agricultural and Food Chemistry*. 53: 2341-2343.

- Long, L., R. Rubin and P. Brodt. 1998. Enhanced invasion and liver colonization by lung carcinoma cells overexpressing the type 1 insulin-like growth factor receptor. *Experimental Cell Research*. 238(1): 116-121.
- Lostao, M.P., B.A. Hirayama, D.D.F. Loo and E.M. Wright. 1994. Phenylglucosides and the Na<sup>+</sup>/glucose cotransporter (SGLT1): Analysis of interactions. *Journal of Membrane Biology*. 142: 161-170.
- Maga, G., and U. Hubescher. 2003. Proliferating Cell Nuclear Antigen (PCNA): a dancer with many partners. *Journal of Cell Science*. 116: 3051-3060.
- Maison, P., B. Balkau, et al. 1998. Growth hormone as a risk for premature mortality in healthy subjects: data from the Paris prospective study. *British Medical Journal*. 316: 1132-1133.
- Matter, W.F., R.F. Brown and C.J. Vlahos. 1992. The inhibition of phosphatidylinositol 3-kinase by quercetin and analogs. *Biochemical and Biophysical Research Communications*. 186(2): 624-631.
- Mawson, A., A. Lai, J.S. Carroll, C.M. Sergio, C.J. Mitchell and B. Sarcevic. 2005. Estrogen and insulin/IGF-I cooperatively stimulate cell cycle progression in MCF-7 breast cancer cells through differential regulation of c-myc and cyclin D1. *Molecular and Cellular Endocrinology*. 229: 161-173.
- Naveh-Many, T., R. Rahamimov, N. Livni and J. Silver. 1995. Parathyroid cell proliferation in normal and chronic renal failure rats. The effects of calcium, phosphate, and Vitamin D. *Journal of Clinical Investigation*. 96(4): 1786-1793.
- Olthof, M.R., P.C.H. Hollman, T.B. Vree and M.B. Katan. 2000. "Bioavailabilities of Q-3-G and Q-4'-G do not differ in humans. *Journal of Nutrition*. 130: 1200-1203.
- Ong, L.C., Y. Jin, I.C. Song, S. Yu, K. Zhang and P.K. Chow. 2008. 2-[<sup>18</sup>F]-2-deoxy-D-glucose (FDG) uptake in human tumor cells is related to the expression of GLUT-1 and hexokinase II. *Acta Radiologica*. 49(10): 1145-1153.

- Papa, V., B. Gliozzo, G.M. Clark, et al. 1993. Insulin-like growth factor-I receptors are overexpressed and predict a low risk in human breast cancer. *Cancer Research*. 53: 3736-3740.
- Peto, J. 2001. Cancer epidemiology in the last century and the next decade. *Nature*. 411: 390-395.
- Plumb, G.W., K.R. Price and G. Williamson. 1999. Antioxidant properties of flavonol glycosides from green beans. *Redox Report*. 4: 123-127.
- Purdue, J.F. 1984. Chemistry, structure, and function of insulin-like growth factors and their receptors: a review. *Canadian Journal of Biochemistry and Cell Biology*. 62: 1237-1245.
- Renvoize, C., A. Biola, M. Pallardy and J. Breard. 1998. Apoptosis: identification of dying cells. *Cell Biology and Toxicology*. 14: 111-120.
- Ribble, D., N.B. Goldstein, D.A. Norris and Y.G. Shellman. 2005. A simple technique for quantifying apoptosis in 96-well plates. *BMC Biotechnology*. 10: 5-12.
- Rogers, S., M.L. Macheda, S.E. Docherty, M.D. Carty, M.A. Henderson, W.C. Soeller and E.M. Gibbs. 2002. Identification of a novel glucose transporter-like protein-GLUT-12. *AJP-Endocrinology and Metabolism*. 282: E733-E738.
- Rogers, S., S.E. Docherty, J.L. Slavin, M.A. Henderson and J.D. Best. 2003. Differential expression of GLUT12 in breast cancer and normal breast tissue. *Cancer Letters*. 193(2): 225-233.
- Sarbassov, D.D., D.A. Guertin, S.M. Ali and D.M. Sabatini. 2005. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science*. 307: 1098-1101.
- Sesink, A.L., K.A. O'Leary and P.C. Hollman. 2001. Quercetin glucuronides but not glucosides are present in human plasma after consumption of quercetin-3-glucoside or quercetin-4'-glucoside. *Journal of Nutrition*. 131(7): 1938-1941.
- Shemer, J., M. Adamo, G.L. Wilson, D. Heffez, Y. Zick and D. LeRoith. 1987. Insulin and insulin-like growth factor-I stimulate a common endogenous

- phosphoprotein substrate (pp185) in intact neuroblastoma cells. *Journal of Biological Chemistry*. 262: 15476-15482.
- Smith, T.A. 1999. Facilitative glucose transporter expression in human cancer tissue. *British Journal of Biomedical Science*. 56: 285-292.
- Smitherman, P.K., A.J. Townsend, T.E. Kute and C.S. Morrow. 2004. Role of multidrug resistance protein 2 (RP2,ABCC2) in alkylating agent detoxification: MRP2 potentiates glutathione S-transferase A1-1-mediated resistance to chlorambucil cytotoxicity. *The Journal Of Pharmacology and Experimental Therapeutics*. 308: 260-267.
- Sun, J., Y. Chu, X. Wu and R.H. Liu. 2002. Antioxidant and antiproliferative activities of common fruits. *Journal of Agricultural and Food Chemistry*. 50: 7449-7454.
- Thissen, J.P., J.M. Ketelslegers and L.E. Underwood. 1994. Nutritional regulation of the insulin-like growth factors. *Endocrine Reviews*. 15: 80-101.
- Tommasi, S. and G.P. Pfeifer. 1999. In vivo structure of two divergent promoters at the human PCNA locus. *Journal of Biological Chemistry*. 274(39): 27829-27838.
- Valentinis, B. and R. Baserga. 2001. IGF-I receptor signalling in transformation and differentiation. *Molecular Pathology*. 54: 133-137.
- Vijayababu, M.R., A. Arunkumar, P. Kanagaraj and J. Arunakaran. 2006. Effects of quercetin on insulin-like growth factors (IGFs) and their binding protein-3 (IGFBP3) secretion and induction of apoptosis in human prostate cancer cells. *Journal of Carcinogenesis*. 5(1): 10.
- Wakeling, A.E., E. Newbould and S.W. Peters. 1989. Effects of antioestrogens on the proliferation of MCF-7 human breast cancer cells. *Journal of Molecular Endocrinology*. 2: 225-234.
- Walgren, R.A., J-T. Lin, R.K-H. Kinne and T. Walle. 2000. Cellular uptake of dietary flavonoid quercetin 4'-beta-glucoside by sodium-dependent glucose transporter SGLT1. *Pharmacology and Experimental Therapeutics*. 294(3): 837-843.

- Wang, S., V.L. DeGroff and S.K. Clinton. 2003. Tomato and soy polyphenols reduce insulin-like growth factor-I-stimulated rat prostate cancer cell proliferation and apoptotic resistance in vitro via inhibition of intracellular signaling pathways involving tyrosine kinase. *Journal of Nutrition*. 133: 2367-2376.
- Weber, G. 1977. Enzymology of cancer cells (second of two parts). *New England Journal of Medicine*. 296: 541-551.
- Weihua, Z., R. Tsan, W-C. Huang, Q. Wu, C-H. Chiu, I.J. Fidler and M-C. Hung. 2008. Survival of cancer cells is maintained by EGFR independent of its kinase activity. *Cancer Cell*. 13(5): 385-393.
- Werner, H. and D. LeRoith. 2000. New concepts in regulation and function of the insulin-like growth factors: implications for understanding normal growth and neoplasia. *Cell and Molecular Life Sciences*. 57: 932-942.
- Willett, W.C. 1995. Diet, nutrition and avoidable cancer. *Environmental Health Perspectives*. 103(S8): 165-170.
- Williamson, G., A.J. Day, G.W. Plumb and D. Couteau. 2000. Human metabolic pathways of dietary flavonoids and cinnamates. *Biochemical Society Transactions*. 28(2): 16-22.
- Wolfe, K.L. and R.H. Liu. 2007. Cellular antioxidant activity (CAA) assay for assessing antioxidants, foods, and dietary supplements. *Journal of Agricultural and Food Chemistry*. 55(22): 8896-8907.
- Xia, Y., L. Jin, B. Zhang, H. Xue, Q. Li and Y. Xu. 2007. The potentiation of curcumin on insulin-like growth factor-1 action in MCF-7 human breast carcinoma cells. *Life Sciences*. 80(23): 2161-2169.
- Xie, Y., B. Skytting, G. Nilsson, B. Brodin and O. Larsson. 1999. Expression of insulin-like growth factor-I receptor in synovial sarcoma: association with an aggressive phenotype. *Cancer Research*. 59: 3588-3591.
- Yakar, S., J.L. Liu, B. Stannard, et al. 1999. Normal growth and development in the absence of hepatic insulin-like growth factor I. *Proceedings of the National Academy of Sciences*. 96: 7324-7329.

- Yen, A., M.S. Roberson, S. Varvayanis and A.T. Lee. 1998. Retinoic acid induced mitogen-activated protein (MAP)/extracellular signal-regulated kinase (ERK) kinase activation needed to elicit HL-60 cell differentiation and growth arrest. *Cancer Research*. 58:3163-3172.
- Yoon, H. and R.H. Liu. 2007. Effect of selected phytochemicals and apple extracts on NF-kB activation in human breast cancer MCF-7 cells. *Journal of Agricultural and Food Chemistry*. 55(8): 3167-3173.
- Youngren, J.F., K. Gable, C. Penaranda, B.A. Maddux, M. Zavodovskaya, M. Lobo and M. Campbell. 2005. Nordihydroguaiaretic acid (NDGA) inhibits the IGF-I and c-erbB2/HER2/neu receptors and suppresses growth in breast cancer cells. *Breast Cancer Research and Treatment*. 94: 37-46.
- Yuhong, L., X. Zi and M. Pollak. 2003. Molecular mechanisms underlying IGF-I-induced attenuation of the growth-inhibitory activity of trastuzumab (Herceptin) on SKBR3 breast cancer cells. *International Journal of Cancer*. 108(3): 334-341.
- Zhang, S. and M.E. Morris. 2003. Effects of the flavonoids biochanin A, morin, phloretin, and silymarin on P-glycoprotein-mediated transport. *Journal of Pharmacology and Experimental Therapeutics*. 304(3): 1258-1267.



## CHAPTER 4

### FRESH APPLES INHIBIT CELL PROLIFERATION AND INDUCE APOPTOSIS IN MAMMARY TISSUES OF RATS IN VIVO

#### ABSTRACT

Apple extracts had potent antioxidant activity and antiproliferative activity against cancer cells *in vitro* and *in vivo*. The objectives of this study were to determine the effects of apple phytochemical extracts on cell proliferation and apoptosis in mammary tissues of rats *in vivo*, and to determine how apple phytochemical extracts affect the expression of cell cycle proteins *in vivo*. Rats were given whole apple extracts (0, 3.3, 10.0 or 20.0 g/kg body weight) by gavage starting 2 weeks after DMBA administration and continuing for 7 weeks. In the apple-treated groups, the expression of proliferating cell nuclear antigen (PCNA), BrdU-labeled cells, Cyclin D1 and Bcl-2 decreased, and Bax expression and nuclear fragmentation increased, suggesting that the dietary intervention effectively protected against chemically-induced mammary carcinogenesis in this model. These results demonstrate the potent capacity of fresh apples to suppress cell proliferation and induce apoptosis in DMBA-treated mammary tissues in rats.

#### INTRODUCTION

Cancer is a serious public health problem worldwide. It results from a multistage, multi-mechanism carcinogenesis process that involves mutagenic, cell death and epigenetic mechanisms with three distinguishable but closely allied stages: initiation, promotion, and progression (Moolgavkar, 1978). In 2008 the American Cancer Society estimated a total of 1,437,180 new cancer cases and 565,650 deaths

from cancers in the United States (Jemal *et al.*, 2008). Although diagnosis and treatment are the major strategies of controlling cancer, cancer prevention is receiving greater attention. Over the past decades, a promising new approach, called “chemoprevention,” which targets the carcinogenic process at the cellular and molecular levels, has been studied. Chemoprevention is the attempt to halt or reverse the development and progression of precancerous cells through use of noncytotoxic nutrients, bioactive compounds, and/or pharmacological agents during the time period between tumor initiation and malignancy (Griffin, 1980). Research has demonstrated that cancer is a largely avoidable disease. It is estimated that more than two-thirds of cancer may be prevented through lifestyle modification (Doll and Peto, 1981; Oliveria *et al.*, 1997; Willett, 2002). Nearly one-third of these cancer occurrences can be attributed to the American diet alone with its typically high-fat, low-fiber content (Doll and Peto, 1981; Willett, 2002). Fruit and vegetable consumption has been consistently shown to reduce the risk of many cancers (Block *et al.*, 1992; Liu *et al.*, 2005; Liu *et al.*, 2009). It has been suggested that the phytochemicals in fruits and vegetables are the primary contributors to the prevention of cancers (Liu, 2003; Liu, 2004).

Apples and apple juice are a very significant part of the diet and are one of the best sources of antioxidant phytochemical compounds in the Western world (Boyer and Liu, 2004; Wolfe *et al.*, 2008). Epidemiological and laboratory studies have shown that consumption of apples and apple juice is associated with a reduced risk of cancer (Deneo-Pellegrini *et al.*, 1996; Gallus *et al.*, 2005; Liu *et al.*, 2005; Michels *et al.*, 2006; Veeriah *et al.*, 2008;). In previous studies, apple extracts have been shown to have potent antioxidant and antiproliferative activity against colon, liver and breast cancer cells *in vitro* in a dose-dependent manner (Eberhardt *et al.*, 2000; Sun *et al.*, 2002; Wolfe *et al.*, 2003; Yoon and Liu, 2007; Maldonado-Celis *et al.*, 2008; Sun and Liu, 2008). While apple extracts inhibited proliferation of estrogen-receptor negative

MDA-MB-231 cells more potently than estrogen-receptor positive MCF-7 cells, the extracts effectively down-regulated Cyclin D1 and CDK-4 expression, decreased Rb phosphorylation, caused G1 cell cycle arrest, and consequently inhibited proliferation in both cell lines (Sun and Liu, 2008). In a previous study, we reported apple extracts had activity inhibiting NF- $\kappa$ B activation in human breast cancer MCF-7 cells (Yoon and Liu, 2007). Recently, we reported that apple extracts inhibited the initiation of mammary carcinogenesis in a DMBA-induced model in rats (Liu *et al.*, 2005; Liu *et al.*, 2009). However, the mechanism(s) of apple phytochemicals in the prevention of cancer is still unclear. Thus, the objectives of the present study were to determine the effects of apple phytochemical extracts on cell proliferation and apoptosis in mammary tissues of rats *in vivo*, and to determine how apple phytochemical extracts affect the expression of cell cycle proteins *in vivo*.

## MATERIALS AND METHODS

**Reagents.** Acetone, wax, ethanol, and formalin (36%) were purchased from Fisher Scientific (Pittsburgh, PA). Hematoxylin, eosin, proteinase K, and 7, 12-dimethylbenz (a)anthracene (DMBA) were obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals used in the study were of analytical grade.

**Apple Extraction.** Apples of the Red Delicious variety were purchased from Cornell Orchards (Cornell University, Ithaca, NY). Fresh apples were cleaned and dried before extraction. Apples were extracted using the method reported previously in our laboratory (Liu *et al.*, 2002; Sun *et al.*, 2002; Liu *et al.*, 2009). Briefly, 100 g fresh weight of the edible part of apples was weighed and homogenized with chilled 80% acetone (1:2, w/v) using a chilled Warning blender for 5 min. The sample was then further homogenized using a Polytron homogenizer for an additional 3 min. The homogenates were filtered through Whatman #1 filter paper on a Buchner funnel

under vacuum. The filtrate was evaporated at 45 °C until approximately 90 % of the filtrate had been evaporated. The apple extracts were standardized to contain  $175.4 \pm 18.8$  mg total phenolics per 100 g apples (Sun *et al.*, 2002). The standardized apple extracts were frozen and stored at -40 °C until use in the feeding study.

**Animal Care and Treatment.** Pathogen-free weanling female Sprague-Dawley rats, 35 days of age, were purchased from Harlan Company (Indianapolis, IN). The rats adapted immediately to the Prolab 5P76 Isopro RMH 3000 diet and were housed in a room for 2 weeks with a 12 h light / 12 h dark cycle prior to the initiation of the experiment. The rats were randomly assigned to five groups (n=12 per group). Four groups of rats (50 days old) were given 10 mg of DMBA dissolved in 1 mL of corn oil by gavage; the fifth group (the negative control group) which received no DMBA was given 1 mL of corn oil. Rats were administered the control extracts or whole apple extracts by gavage, starting 2 weeks after DMBA administration and continuing for the 7 weeks until the end of the experiment. The apple treatments were 3.3 (low), 10.0 (middle) and 20.0 (high) g fresh apples/kg body weight, which were equivalent to human consumption of 1, 3, and 6 apples per day, respectively. Animals were weighed weekly.

All animals were sacrificed under anesthesia at the end of the 9<sup>th</sup> week after DMBA administration and arterial blood samples were collected from each rat. At least 2 h prior to killing, rats were injected with 5-bromo-2'-deoxyuridine (BrdU) at a dose of 50 mg/kg body weight, intraperitoneally for the mammary BrdU-labeling index. All mammary glands were removed, fixed in 70 % alcohol, and subsequently dehydrated and embedded in wax. The tissue wax was cut into 3 µm sections, fixed on slides and processed for immunohistochemistry analysis.

**BrdU Incorporation Assay.** Cells in mammary gland tissues undergoing DNA synthesis were labeled with BrdU and analyzed using a ZYMED BrdU Staining Kit

(Invitrogen, South San Francisco, CA). Specimens were dewaxed and immersed in PBS (phosphate-buffered saline) containing 3.0 % hydrogen peroxide for 10 min at room temperature. The sections were washed twice in distilled water for 5 min (each) at room temperature and then washed three times with PBS. The trypsin solution was applied directly onto the specimens, which were incubated for 10 min. After the sections had been washed three times with PBS, they were submerged in denaturing solution for 30 min, washed three times with PBS, and incubated in Blocking Solution for 10 min. A sufficient amount of Biotinylated Mouse Anti-BrdU solution was applied directly onto the specimens, which were incubated for 1 h at 37 °C in a humidified chamber. After the sections had been washed three times with PBS, an amount of peroxidase-anti-mouse IgG2a solution sufficient to cover the specimens was added and sections were incubated for 10 min at room temperature. The chromogenic reaction was developed with 3, 3'-diaminobenzidine (DAB) for 3 min, and all sections were counterstained with hematoxylin. A minimum of 4,000 cells in each tissue sample were randomly selected for the BrdU incorporation assay, and positive cells were counted.

**Immunohistochemical Staining of PCNA, Cyclin D1, Bcl-2, and Bax.** The sections of mammary gland tissues were deparaffinized in xylene and rehydrated through graded alcohol. The sections were incubated for 10 min at 95 - 100 °C in 10 mmol/L sodium citrate buffer (pH 6.0). Endogenous peroxidases in the mammary gland cells were inactivated by immersing the sections in 0.3% hydrogen peroxide for 10 min, and the sections were then incubated for 10 min with 10 % normal goat serum to block non-specific binding. The sections were subsequently incubated at 4 °C overnight with one of four antibodies: 1) anti-PCNA antibody (monoclonal mouse, PC10, IgG, 1:50 dilution, Calbiochem, Lab, Inc., Temecula, CA); 2) anti-cyclin D1 antibody (monoclonal mouse, DCS-6, IgG, 1:25 dilution, Calbiochem, Lab, Inc.,

Temecula, CA); 3) anti-Bcl-2 antibody (rabbit polyclonal, N-9, IgG, 1:15 dilution, Santa Cruz Biotechnology, Delaware, CA); or 4) anti-Bax antibody (polyclonal rabbit, AB-1, IgG, 1:20 dilution, Calbiochem, Lab, Inc., Temecula, CA). The sections were then incubated with biotinylated anti-mouse IgG or anti-rabbit IgG (ZYMED, Lab, Inc., Carlsbad, CA) for 30 min, followed by peroxidase-conjugated streptavidin (ZYMED, Lab, Inc) for 30 min. The chromogenic reaction was developed with DAB for 3 min, and all sections were counterstained with hematoxylin. The same protocol was applied to the controls with the omission of the primary antibody. Microscopic images were measured at a magnification of 400 $\times$ . Two thousand cells were counted in five visual fields in one of 4 glands from each rat, and an average of the cells positively stained for specific protein expression (mean  $\pm$  S.D.) in mammary gland tissues randomly chosen from 8 rats in each group were analyzed.

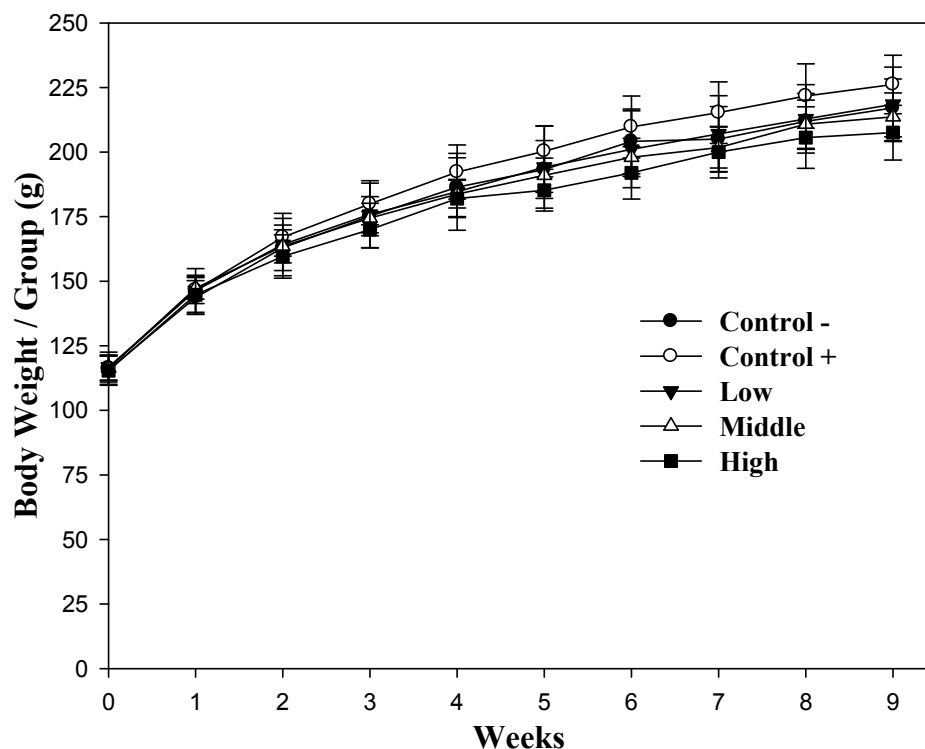
**Immunohistochemical Staining by TUNEL Assay.** Cell apoptosis was determined using the ApopTag@Plus Peroxidase *In Situ* Apoptosis Detection Kit (Serologicals Corporation, Norcross, GA) based on the terminal deoxynucleotidyl-transferase mediated dUTP nick end labeling (TUNEL) assay (Liu *et al.*, 2009). Briefly, the slices of rat mammary gland tissues were deparaffinized in xylene and rehydrated through graded alcohol, then washed in PBS, treated with proteinase K (20  $\mu$ g/ml) for 15 min at room temperature, quenched in 3.0 % hydrogen peroxide in PBS for 5 min, washed 2 times in PBS, incubated in equilibration buffer at least 10 seconds, and labeled with TdT reaction mix in a humidified box for 1 h at 37  $^{\circ}$ C. The slices were washed in stop/wash buffer for 10 min and then washed 3 times in PBS. The slides were then incubated for 30 min in a solution of anti-Digoxigenin Conjugate, and colorized with DAB. All sections were counterstained with 0.5 % (w:v) methyl green. Controls consisted of omission of the TdT reaction mix. Microscopic images were measured at a magnification of 400 $\times$ . Four thousand cells

were counted in five visual fields randomly in the section, and mammary gland tissues randomly chosen from 8 rats in each group were examined to determine the number of apoptotic cells (mean  $\pm$  S.D.).

**Statistical Analysis.** Data were expressed as mean  $\pm$  S.D. Levene's test for equality of variances followed by the *t*-test for equality of means were used to analyze body weight. The expression of PCNA, TUNEL, Cyclin D1, Bcl-2, Bax, and BrdU labeling in mammary gland tissues were analyzed using the Student's *t*-test, Welch's *t*-test or ANOVA. Data analyses were generated and plots were constructed using SPSS for Windows version 16.0 (SPSS Inc., Chicago, IL) and SigmaPlot version 11 for Windows (Systat Software Inc., San Jose, CA). Statistical significance was set at  $P < 0.05$ , and all *P* values were unadjusted for multiple comparisons.

## RESULTS

**Effect of Apple Extracts on Body Weight.** Body weights of animals in each group were monitored weekly and are presented in Figure 4.1. Throughout the duration of the experiment, the body weights of the low, middle and high apple groups were not statistically different from one another. Similarly, the body weights of the positive and negative control groups did not differ from each other throughout the experiment. At the end of week 9, both the negative ( $217 \pm 11$  g) and the positive ( $226 \pm 11$  g) control groups weighed more than the high apple group ( $208 \pm 17$  g) ( $P < 0.05$ ). The positive control group outweighed the middle apple group ( $214 \pm 9$  g) as well. Body weights were within a normal range, suggesting no toxicity in the animals fed apple extracts as reported previously (Liu *et al.*, 2009).

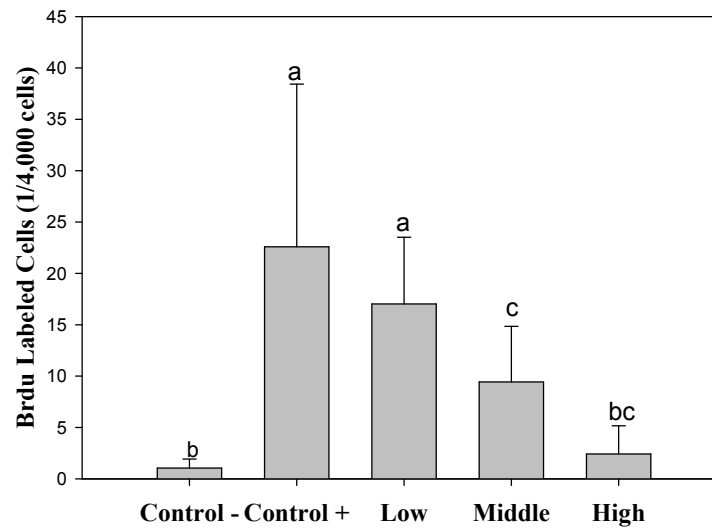
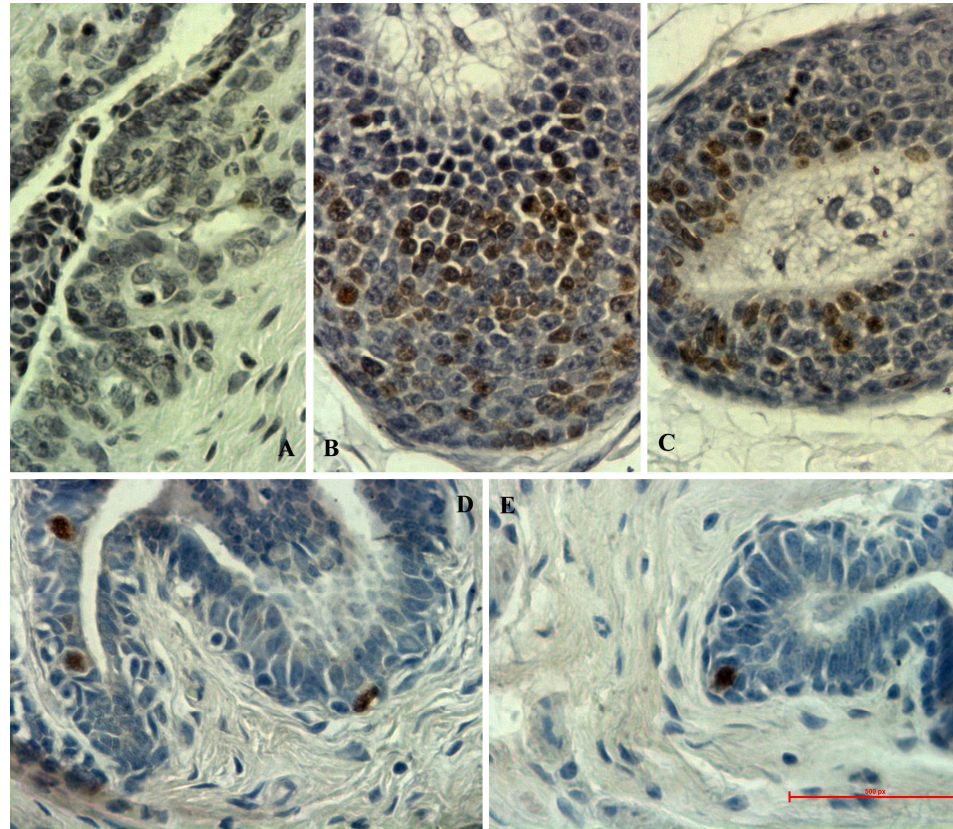


**Figure 4.1. Effects of varying doses of apple extracts on body weight of rats.** The female Sprague-Dawley rats were fed 3.3 (low), 10.0 (middle), or 20.0 (high) g fresh apples/kg body weight starting 2 weeks after DMBA administration and continuing for 7 weeks until the end of the experiment. Control -: the negative control group; Control +: the positive control group.

#### **Immunohistochemistry for BrdU Labeling in Mammary Gland Tissue.**

Cell proliferation in mammary gland tissues, as measured by nuclear BrdU incorporation, was significantly lower in the apple treatment groups than in the positive control group in a dose dependent manner ( $P<0.05$ ) (Figure 4.2). A low rate of BrdU incorporation was observed in the negative control group ( $1.1 \pm 0.8$ , per 4,000 cells). A high rate of BrdU incorporation was observed in the positive control group ( $22.6 \pm 15.8$ , per 4,000 cells). The inhibitory rates of BrdU incorporation in mammary gland tissues were 24.6, 58.2 ( $P<0.05$ ), and 89.3 % ( $P<0.01$ ) in the low, middle, and high dose groups, respectively, compared to the positive control group.





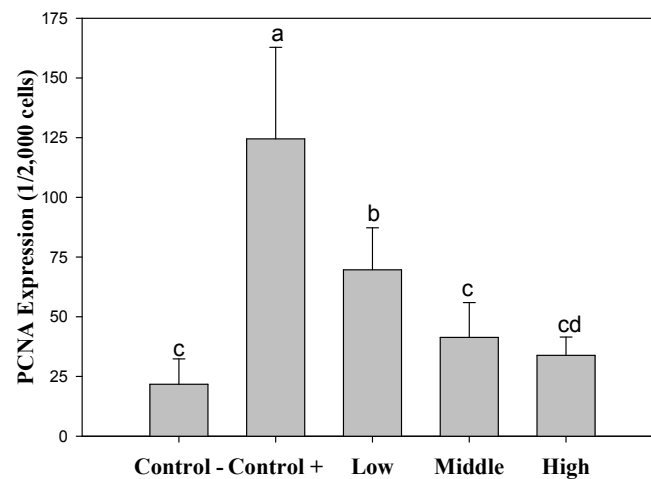
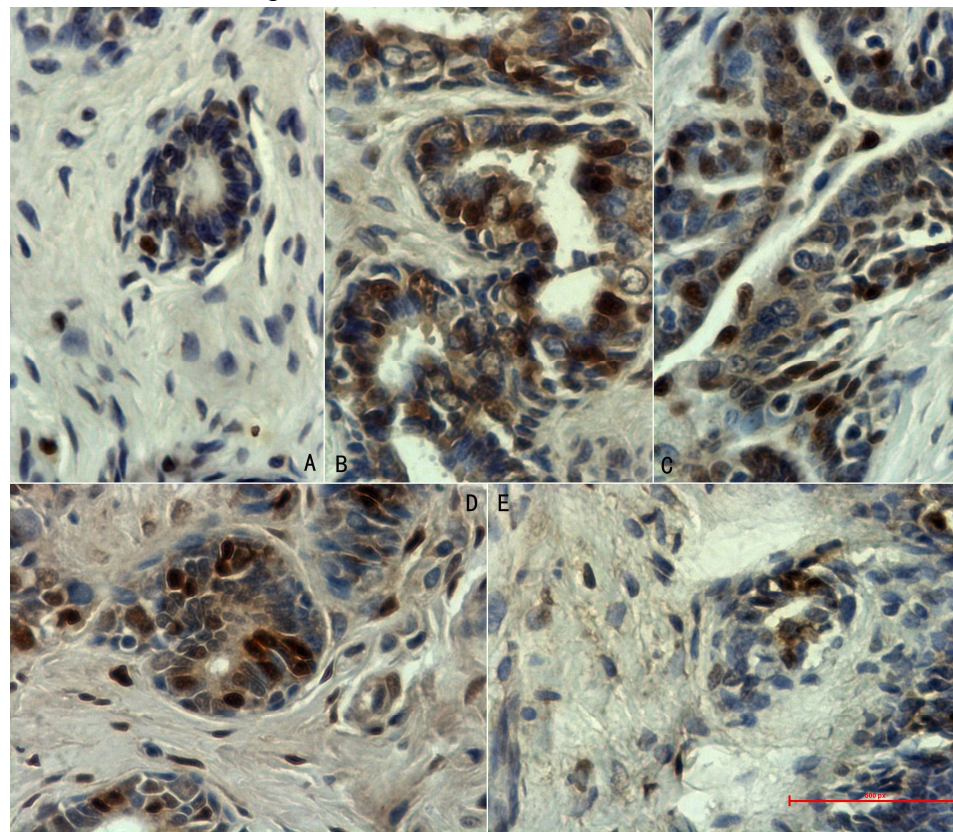
**Figure 4.2. Effects of varying doses of fresh apple extracts on BrdU labeling in rat mammary gland cells *in vivo*.** High power view (400×) of the cells of BrdU labeling: (A) negative control group (Control -) (n=10); (B) positive control group (Control +) (n=10); (C) low dose (n=9); (D) middle dose (n=10); and (E) high dose (n=10). The BrdU labeled cells were visualized with DAB; positive cells are reddish-brown in color. Bars with no letters in common are significantly different ( $P<0.05$ ).

**Effects of Apple Extracts on the Cell Cycle and Related Proteins in Mammary Gland Tissue.** As shown in Figure 4.3, the expression of proliferating cell nuclear antigen (PCNA) was the lowest in the negative control group. With DMBA treatment, the expression of PCNA was dramatically increased when compared to the negative control ( $P<0.05$ ). PCNA expression in the apple-treated groups was significantly lower than that in the positive control group ( $P<0.05$ ) (Figure 4.3). Compared with the positive control group, the inhibitory rates of PCNA expression were 44.0, 66.8, and 72.8 % in the low, middle, and high dose groups, respectively, demonstrating a clear dose response.

Cyclin D1 is one of the key markers in the G1 to S phase transition of the cell cycle. Cyclin D1 expression was detected among the mammary gland tissues in the control and apple-treated groups by immunohistochemistry. As shown in Figure 4.4, a low expression of Cyclin D1 in mammary gland tissues from the negative control group was observed. Cyclin D1 expression significantly decreased in the apple-treated groups compared to the positive control group ( $30.7 \pm 12.3$ , per 4,000 cells;  $P<0.05$ ) (Figure 4.4). A dose response was also observed. The inhibitory rates of Cyclin D1 expression were 22.1, 58.5, and 73.5 % in the low, middle, and high doses of apple extracts, respectively, in comparison with the positive control group.

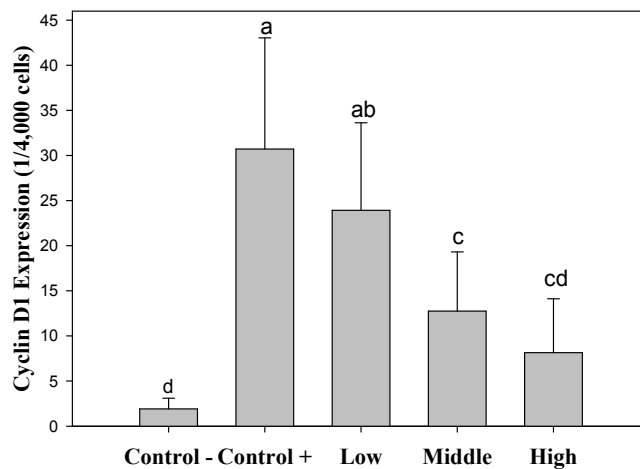
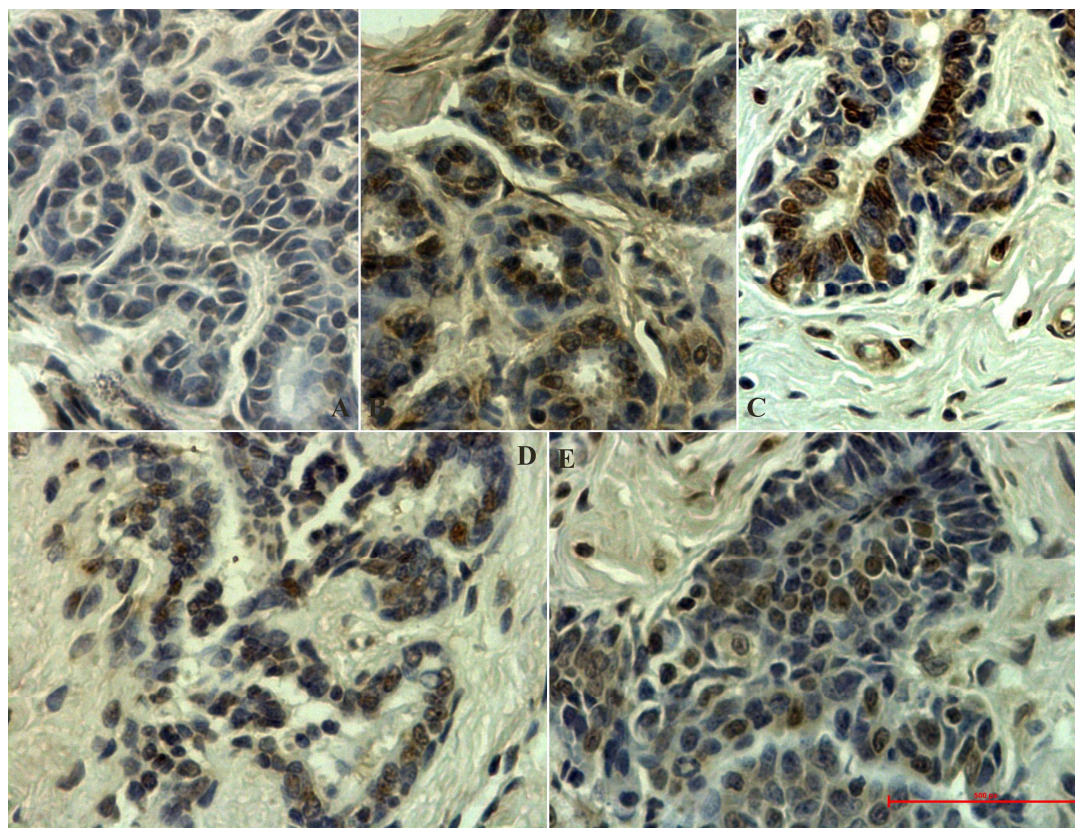
**Apoptosis Induction and Expression of Related Proteins in Mammary Gland Tissue.** Apoptosis was detected in mammary gland tissues using the TUNEL assay. As shown in Figure 4.5, a low rate of apoptosis was observed in the mammary gland tissues from the negative control group ( $4.9 \pm 2.3$ , per 4,000 cells). Occurrences of apoptosis in mammary gland cells in the apple treatments were significantly higher than those in the control groups ( $P<0.01$ ). Apoptosis expression was increased by 6.1, 12.9 and 14.6 times in the low, middle, and high dose groups, respectively, compared

to the positive control group ( $2.9 \pm 1.3$ , per 4,000 cells). Apoptosis was induced by apple extracts in a dose-dependent manner.

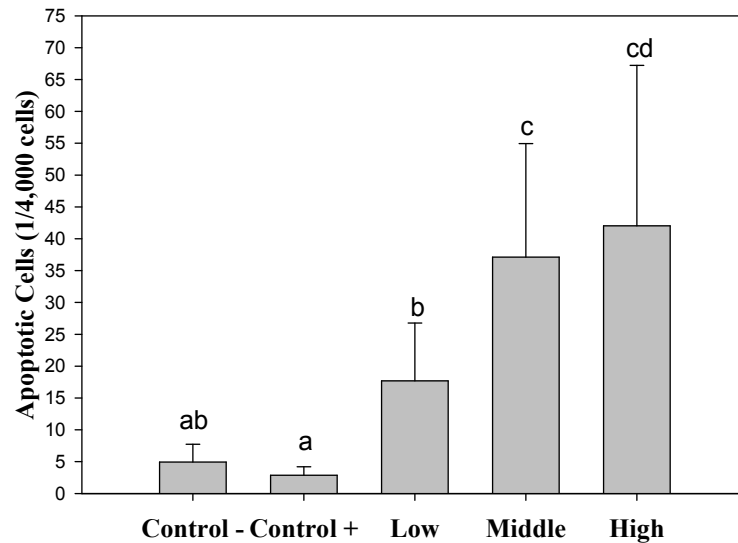
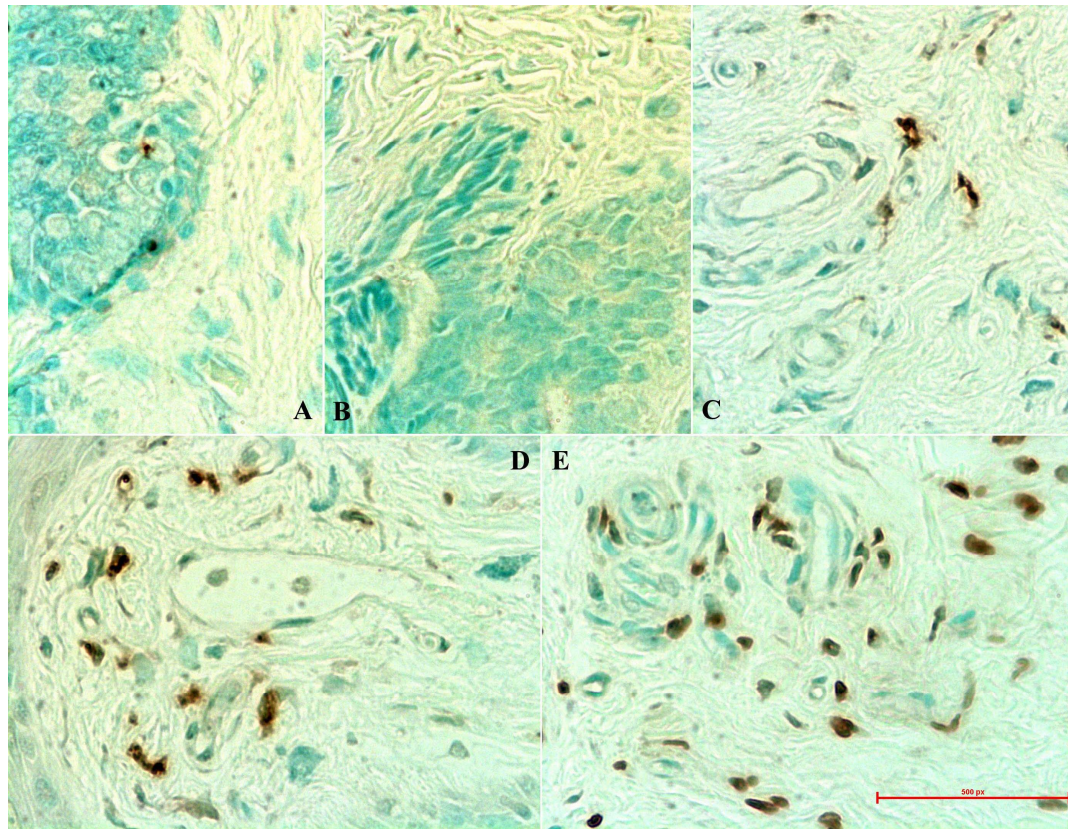


**Figure 4.3. Effects of varying doses of fresh apple extracts on PCNA expression in rat mammary gland tissues *in vivo*.** High power view (400 $\times$ ) of the PCNA expression: (A) negative control group (Control -) (n=8); (B) positive control group (Control +) (n=8); (C) low dose (n=8); (D) middle dose (n=8); and (E) high dose (n=8). The expression of PCNA was visualized with DAB; positive cells are reddish-brown in color. Bars with no letters in common are significantly different ( $P < 0.05$ ).





**Figure 4.4. Effects of varying doses of fresh apple extracts on expression of Cyclin D1 in rat mammary gland tissues *in vivo*.** High power view (400×) of Cyclin D1 expression: (A) negative control group (Control -) (n=8); (B) positive control group (Control +) (n=8); (C) low dose (n=8); (D) middle dose (n=8); and (E) high dose (n=8). The expression of Cyclin D1 was visualized with DAB; positive cells are reddish-brown in color. Bars with no letter in commons are significantly different ( $P<0.05$ ).



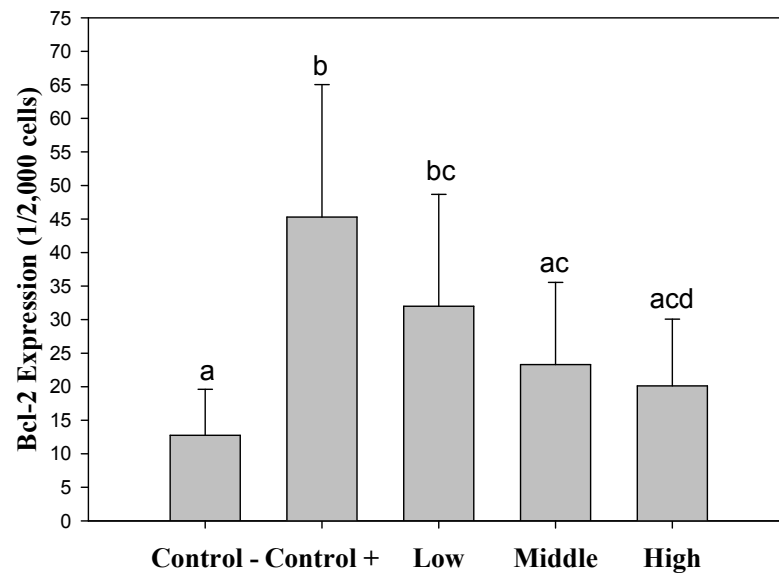
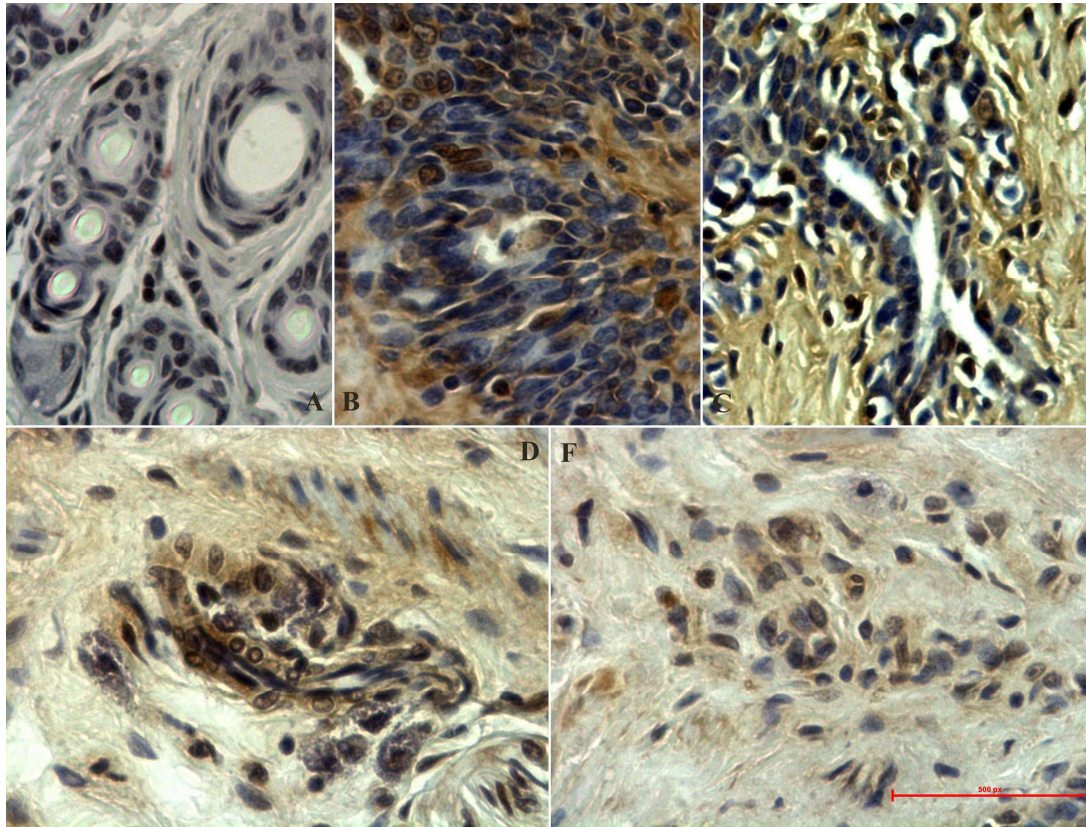
**Figure 4.5. TUNEL Assay: Effects of varying doses of fresh apple extracts on induction of apoptosis in rat mammary gland tissues *in vivo*.** High power view (400×) of apoptosis expression: **(A)** negative control group (Control -) (n=8); **(B)** positive control group (Control +) (n=8); **(C)** low dose (n=8); **(D)** middle dose (n=8); and **(E)** high dose (n=8). The apoptotic cells were visualized with DAB; positive cells are reddish-brown in color. Bars with no letters in common are significantly different ( $P<0.05$ ).

The expression of Bcl-2 and Bax was examined in mammary gland tissues among the control and apple-treated groups by immunohistochemistry. Both Bcl-2 and Bax expression in the mammary gland tissues were observed in the negative control group. Bcl-2 expression in the apple treated groups was significantly lower than that in the positive control group in a dose dependent manner ( $P<0.05$ ) (Figure 4.6). The inhibitory rates of Bcl-2 expression were 29.3, 48.6, and 55.6% in the low, middle, and high dose groups, respectively, in comparison with the positive control group ( $45.3 \pm 19.8$ , per 2,000 cells). Bax expression in the positive control group ( $7.0 \pm 2.7$ , per 2,000 cells) was significantly lower than that in the apple-treated groups ( $P<0.05$ ) (Figure 4.7). Bax expression in the negative control group was  $8.5 \pm 5.6$ , per 2,000 cells. The expression of Bax in the lowest dose group ( $15.9 \pm 6.1$ , per 2,000 cells) was 2.3 times greater than that in the positive control group. A dose response relationship was observed among the apple-treated groups as compared to the positive control group.

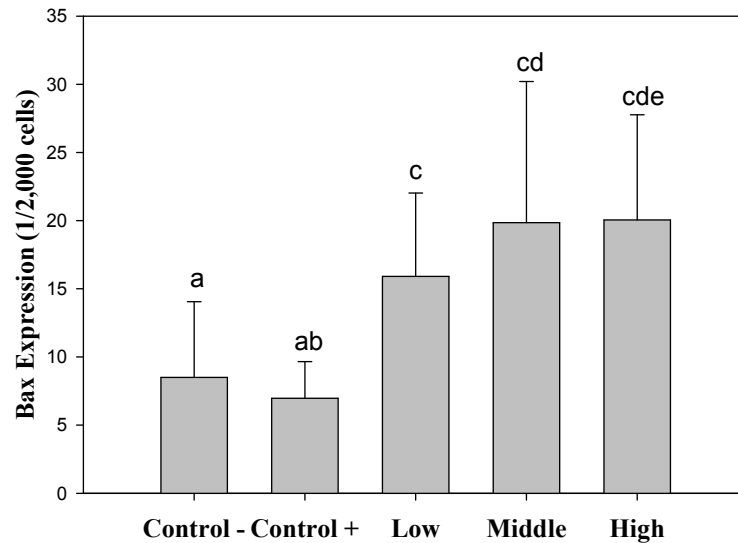
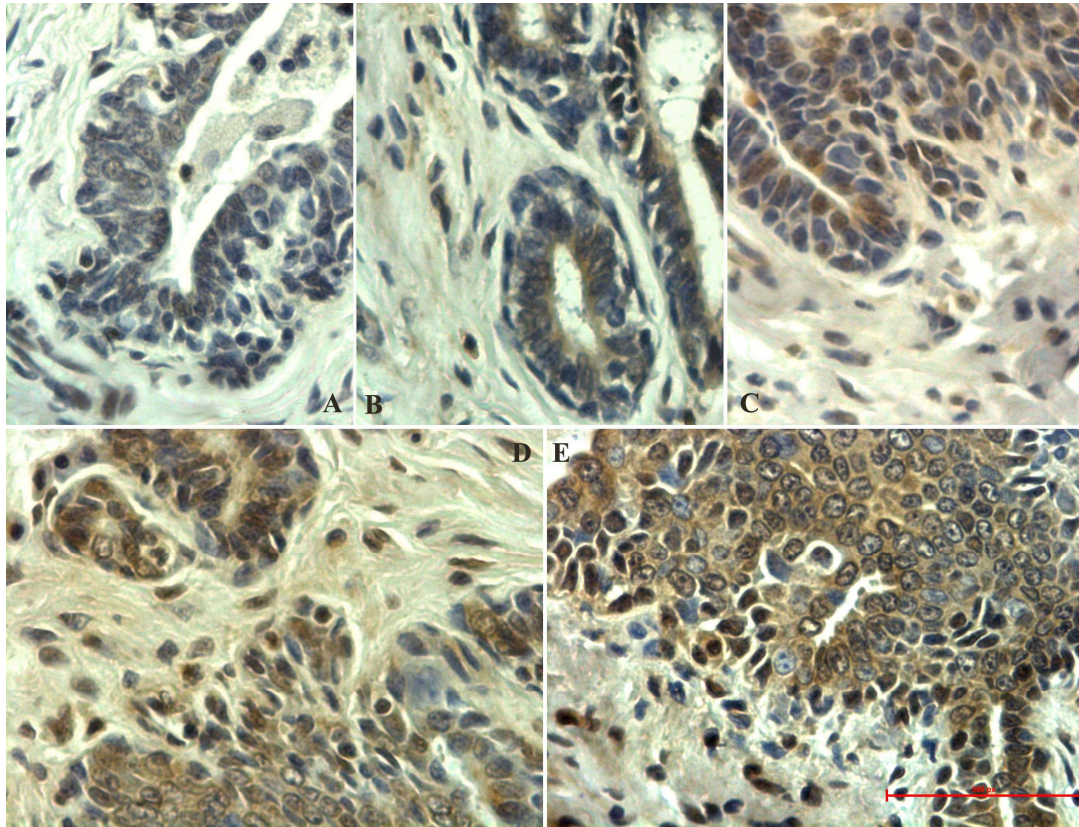
## DISCUSSION

Epidemiological studies, including case-control and cohort studies, have consistently shown that regular consumption of fruits and vegetables is associated with a reduced risk of developing cancer and other chronic diseases (Block *et al.*, 1992). In a large cohort study of 34,467 women in the Nurses' Health Study (NHS) who had undergone colonoscopy or sigmoidoscopy, it was found that consumption of fruit was inversely related to the risk of distal adenomas of the colon or rectum after controlling for potential confounding factors (Michels *et al.*, 2006). A case-control study in Uruguay has shown that apple consumption was associated with significant reduction in colorectal cancer risk for men and women (Deneo-Pellegrini *et al.*, 1996). Other case-control studies conducted in Italy have shown that there is a consistent inverse





**Figure 4.6.** Effects of varying doses of fresh apple extracts on Bcl-2 expression in rat mammary gland tissues *in vivo*. High power view (400×) of Bcl-2 expression in the following groups: (A) negative control group (Control -) (n=8); (B) positive control group (Control +) (n=8); (C) low dose (n=8); (D) middle dose (n=8); and (E) high dose (n=8). The cell plasma was visualized with DAB; positive cells are reddish-brown in color. Bars with no letters in common are significantly different ( $P < 0.05$ ).



**Figure 4.7. Effects of varying doses of fresh apple extracts on Bax expression in rat mammary gland tissues *in vivo*.** High power view (400 $\times$ ) of Bax expression in the following groups: **(A)** negative control group (Control -) (n=8); **(B)** positive control group (Control +) (n=8); **(C)** low dose (n=8); **(D)** middle dose (n=8); and **(E)** high dose (n=8). The cell plasma was visualized with DAB; positive cells are reddish-brown in color. Bars with no letters in common are significantly different ( $P < 0.05$ ).



association between apples and risk of various cancers. The studies included cases of various types of cancer: 598 of the oral cavity and pharynx, 304 of the esophagus, 460 of the larynx, 1,953 of the colorectum, 2,569 of the breast, 1,031 of the ovary and 1,294 of the prostate (Gallus *et al.*, 2005). A case-control study in Brazil found regular apple consumption to be associated with reduced breast cancer risk (OR = 0.30) after adjustment for family income level, since income was also found to have both a strong association with breast cancer and an influence on diet (Di Pietro *et al.*, 2007).

In previous studies of selected common fruits and vegetables, apple has been shown to possess potent antioxidant and anti-proliferative activity against cancer cells (Chu *et al.*, 2002; Sun *et al.*, 2002). Median effective doses (EC<sub>50</sub>) were  $42.5 \pm 2.6$  mg/mL and  $49.4 \pm 1.6$  mg/mL in human colon cancer Caco-2 cells and liver cancer HepG<sub>2</sub> cells treated with different levels of whole apple extracts (Liu and Sun, 2003).

Slattery (2001) discusses the strengths and limitations that the various types of epidemiology studies (clinical, case-control, and cohort) have in determining the association between diet and cancer. She underscores the importance of timing and duration of exposure, considering that certain foods such as fruits and vegetables may exert protective effects at a specific stage in the disease process. In that light, animal and cell studies become extremely valuable in helping to uncover the mechanisms by which whole foods or individual phytochemicals can prevent or slow the growth of cancer. That information, in turn, is crucial for effective and careful design of dietary intervention studies.

In our previous study, apple extracts inhibited mammary carcinogenesis in a DMBA-induced rat mammary cancer model during a 24-week study (Liu *et al.*, 2005; Liu *et al.*, 2009). A dose-response relationship was observed. Apple extracts not only inhibited cell proliferation in mammary tumor tissues, but also induced apoptosis in the mammary tumor tissues from this rat model. The inhibition of cell proliferation in

mammary tumor cells was attributed to down-regulation of PCNA, Cyclin D1 and Bcl-2 and up-regulation of Bax and nuclear fragments (Liu *et al.*, 2009).

Several major groups of polyphenolic compounds occur in apples: flavan-3-ols, flavonols, dihydrochalcones, anthocyanins, and phenolic acids (hydroxybenzoic acid and hydroxycinnamic acids) (Tsao *et al.*, 2003). The major flavan-3-ols, or catechins, present in apples are catechin and epicatechin (stereoisomers), procyanidin B1 (dimer of epicatechin and catechin) and procyanidin B2 (dimer of two epicatechin molecules). A study by Gosse *et al.* (2005) reported that apple procyanidins in drinking water prevented colon carcinogenesis in male Wistar rats induced by azoxymethane (AOM). Gosse *et al.* (2006) also reported that apple procyanidins had chemopreventive properties in an *in vitro* model of colon cancer, affecting intracellular signaling pathways and triggering apoptosis in a human adenocarcinoma-derived metastatic cell line (SW620).

The second major group of polyphenols in apples is the flavonols, such as quercetin, kaempferol and myricetin. However, quercetin and its glycosides, occurring nearly exclusively in the peel, are by far the most abundant of the flavonoids and can contribute up to 18% of total phenolics in the apple (Tsao *et al.*, 2003). In our lab, 29 compounds were isolated from red delicious apple peels, including triterpenoids, flavonoids, organic acids, and plant sterols (He and Liu, 2008). Quercetin-3-O- $\beta$ -D-glucopyranoside was one of major flavonoids in the peel, followed by quercetin-3-O- $\beta$ -D-galactopyranoside. Trace amounts of quercetin were detected along with (-)-catechin, (-)-epicatechin, and quercetin-3-O- $\alpha$ -L-arabinofuranoside. Quercetin exhibited anti-proliferative activity against MCF-7 cells *in vitro* with an EC<sub>50</sub> of  $137.5 \pm 2.6 \mu\text{M}$ , while the quercetin-3-O- $\beta$ -D-glucopyranoside was even more potent with an EC<sub>50</sub> of  $23.9 \pm 3.9 \mu\text{M}$ .

The anti-cancer properties of quercetin have been extensively studied *in vitro*. For example, quercetin induced a G1 cell cycle arrest and apoptosis in MDA-MB-453 mammary carcinoma cells via down-regulation of Bcl-2 expression and up-regulation of Bax expression and cleavage of caspase-3 and PARP (Choi *et al.*, 2008). In MDA-MB-468 human breast cancer cells, quercetin reduced proliferation and blocked cell cycle progression by inhibiting the expression of a mutant p53, the form highly expressed in that cell line, at the translational level (Avila *et al.*, 1994). Her-2/*neu*, a receptor tyrosine kinase (RTK) that functions through dimerizing with other RTKs, is an important player in breast cancer signaling (Hynes and Lane, 2005; Olayioye *et al.*, 2000). Quercetin caused down-regulation of the Her-2/*neu* receptor, along with a corresponding decrease in phosphorylation of PI3K and Akt, in human breast carcinoma SK-Br3 cells which over-express Her-2/*neu* (Jeong *et al.*, 2008). A similar down-regulation of the Her-2/*neu* receptor by quercetin in MCF-7 cells, and in a Her-2/*neu* over-expressing ovarian cancer cell line SK-Ov3 was observed. Quercetin has been shown to interfere with signaling pathways involved in migration and metastasis. Matrix metalloproteinases are responsible for dissolving the extracellular matrix, allowing tumor cells to migrate and invade distant sites (Duffy *et al.*, 2000). TPA (12-O-tetradecanoylphorbol-13-acetate) induces MMP-9 through a protein kinase C (PKC) $\delta$ /extracellular signal-regulated kinase (ERK)/AP-1 pathway. In MCF-7 cells, quercetin blocked this induction by reducing activation of PKC $\delta$  and ERK, and reducing AP-1 activity (Lin *et al.*, 2008). As a result, MMP-9 expression, and cell migration and invasion were reduced.

In this study, female Sprague-Dawley rats were fed 3.3 (low), 10.0 (middle), or 20.0 (high) g fresh apples/kg body weight per day starting 2 weeks after DMBA administration and continuing for 7 weeks until the end of the experiment. Body weights in the low, middle and high apple groups were all statistically the same, yet

the markers of proliferation and apoptosis were clearly different in a dose-dependent manner. In addition, the body weights of the negative and positive control groups were statistically the same throughout the experiment, and yet the markers were dramatically different. By the end of the experiment (week 9), the positive control group weighed more than high and middle apple groups and had the expected corresponding higher proliferation and lower apoptosis markers than the high and middle apple groups. On the other hand, by week 9, the negative control group weighed more than the high apple group but had proliferation markers that were somewhat lower than, yet statistically the same as, the high apple group. There was no consistent pattern within this experiment to indicate that body weights influenced the proliferation and apoptosis of mammary cells in these animals.

Cell proliferation in the mammary gland tissues was determined using the BrdU-labeling assay and PCNA expression as the proliferative markers. Apple extracts significantly inhibited the expression of PCNA and BrdU incorporation in mammary gland tissues compared to the positive control group in a dose-dependent manner ( $P<0.05$ ) (Figures 4.2 and 4.3). This indicates that apple extracts could inhibit cell proliferation and DNA synthesis in the mammary gland tissues of DMBA-treated rats. BrdU, a thymidine analogue incorporated into DNA, can be quantified through fluorescent or chromophoric quenching of dyes which bind to DNA or through antibodies to BrdU (Gratzner, 1982). The BrdU incorporation into cells as a proliferative marker is an early and sensitive index for *in vivo* studies. A number of therapeutic agents have been observed to reduce BrdU-labeling in mammary tumor cells in a DMBA-induced mammary model (Ip *et al.*, 1994; Kitagawa and Noguchi, 1994; Whitsett *et al.*, 2006; Liu *et al.*, 2009). PCNA is a nuclear protein which functions as an auxiliary protein for DNA polymerase  $\delta$  and is an absolute requirement for DNA synthesis. PCNA, a key protein in DNA replication and DNA damage repair,

is a stable cell-cycle regulated nuclear protein that is expressed during the cell cycle and whose rate of synthesis is correlated directly with the proliferative rate of cells (Bravo *et al.*, 1987). The higher specificity of PCNA for the S-phase might be thought to be advantageous over other proliferation markers as it should label those cells that have passed the important G1/S boundary of the cell cycle. PCNA has been shown to be more sensitive in detecting proliferating cells in some tumors (Fairman, 1990; Hall and Levinson, 1990; McCormick and Hall, 1992). In this study, very low levels of PCNA expression and BrdU-labeled cells were observed in the negative control group, and both were highly expressed in the DMBA-treated (positive control) group. This indicates that PCNA expression and BrdU-labeled cells are sensitive markers of cell proliferation of mammary gland tissues. The apple extracts reduced both markers in a dose-dependent manner indicating the extracts may inhibit cell proliferation in the mammary tissues of DMBA-treated rats.

In the present study, our findings showed that apple extracts not only affected the expression of a major protein (Cyclin D1) in mammary gland tissues, but also induced apoptosis and the expression of related proteins. The results showed that apple extracts significantly inhibited Cyclin D1 expression and significantly increased apoptosis compared to the positive control group. Cell proliferation of mammary cancer cells is tightly mediated through cell cycle control (Sanchez and Dynlacht, 1996). The deregulation of the cell cycle has been reported to be correlated with the induction of apoptosis (Eastman and Rigas, 1999). The cyclin-dependent kinases (CDKs) are key cell cycle regulators, and their activities are modulated by binding to cyclins (Helin, 1998). Binding of Cyclin D to CDK4 and CDK6 leads to the phosphorylation of the retinoblastoma (Rb) protein. Phosphorylation of Rb prevents it from repressing the E2F family of transcription factors and leads to the transcription of several genes required for the G1-to-S phase transition, thereby promoting cellular

proliferation (Sherr, 1995). Cyclin D1 is over-expressed in 35-50% of breast cancers (Ishii *et al.*, 2006). Over-expression of Cyclin D1 has also been linked to the development of endocrine resistance in breast cancer cells (Hui *et al.*, 2002). A number of therapeutic agents have been observed to induce Cyclin D1 degradation *in vitro* (Langenfeld *et al.*, 1997; Huang *et al.*, 2005). These studies indicate that the induction of Cyclin D1 degradation might offer a useful avenue for therapeutic intervention (Yu *et al.*, 2001). In our previous study, apple extracts inhibited Cyclin D1 expression in mammary tumor tissue in a 24-week study (Liu *et al.*, 2009). Significantly decreased Cyclin D1 expression was also found in the mammary gland tissues in the apple-treated groups of the current study. This indicates that apple extract not only affects the cell cycle during initiation, but it also affects proliferation of cells post-initiation in the DMBA-induced mammary carcinogenesis rat model.

In contrast to non-specific cellular necrosis, apoptosis is characterized by a specific pattern of DNA degradation that exposes 3'-OH ends. As a result, apoptotic cells within tissue sections can be identified by the terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine nick end labeling (TUNEL) of exposed 3'-OH ends with non-isotopically digoxigenin-labelled dUTP (Huerta *et al.*, 2007; Gavrieli *et al.*, 1992). The TUNEL assay is widely used and accepted as a tool for indicating apoptosis. Bcl-2 family proteins are mainly involved in the cell-intrinsic apoptotic pathway by activation of pro-apoptotic Bcl-2 family members, which induce the permeabilization of the outer mitochondrial membrane (OMM), resulting in the release of cytochrome-*c* (Cyt-*c*) and other inter-membrane space proteins (Roussi *et al.*, 2007). Functionally, the Bcl-2-related proteins either inhibit or promote apoptosis, and interactions among these proteins determine whether a cell lives or dies. Bcl-2 is an anti-apoptotic protein and Bax a pro-apoptotic protein. In our study, apple extracts induced apoptosis in mammary gland tissues from the apple-treated groups. In

addition, apple extracts significantly decreased Bcl-2 expression and increased Bax expression in the apple-treated groups in a dose dependent manner. Thus, we can partly explain the mechanism by which apple extracts induce apoptosis: via regulation of the Bcl-2 pathway by down-regulation of Bcl-2 expression and up-regulation of Bax expression in mammary gland tissue.

In conclusion, our data demonstrated that whole apple extracts potentially inhibited proliferation and induced apoptosis in mammary tissues of DMBA-treated Sprague-Dawley rats. These results suggest that a dietary intervention effectively protected against chemically-induced mammary carcinogenesis in this model. The inhibition of cell proliferation and induction of apoptosis in mammary cancer may be regulated through the down-regulation of Cyclin D1 and Bcl-2 expression as well as up-regulation of Bax expression. Thus, these findings strongly support the view that consumption of whole apples is an effective way to achieve cancer protection as well as support our previous hypothesis that health benefits of fruits and vegetables are due to the additive and/or synergistic effects of phytochemicals (Liu, 2004). Nevertheless, the exact mechanism(s) of how apples prevent mammary cancer warrants further study.

## REFERENCES

- Avila, M.A., J.A. Velasco, J. Cansado and V. Votario. 1994. Quercetin mediated the down-regulation of mutant p53 in the human breast cancer cell line MDA-MB468. *Cancer Research*. 54: 2424-2428.
- Block, G., B. Patterson and A. Subar. 1992. Fruit, vegetables, and cancer prevention: a review of the epidemiological evidence. *Nutrition and Cancer*. 18(1): 1-29.
- Boyer, J. and R.H. Liu. 2004. Apple phytochemicals and their health benefits. *Nutrition*. J. 3:5.
- Bravo, R., R. Frank, P.A. Blundell and H. Macdonald-Bravo. 1987. Cyclin/PCNA is the auxiliary protein of DNA polymerase-delta. *Nature*. 326(6112): 515-517.
- Choi, E.J., S.M. Bai and W.S. Ahn. 2008. Antiproliferative effects of quercetin through cell cycle arrest and apoptosis in human breast cancer MDA-MB-453 cells. *Archives of Pharmacal Research*. 31(10): 1281-1285.
- Chu, Y.F., J. Sun, X. Wu and R.H. Liu. 2002. Antioxidant and antiproliferative activities of common vegetables. *Journal of Agricultural and Food Chemistry*. 50(23): 6910-6916.
- Deneo-Pellegrini, H., E. De Stefani and A. Ronco. 1996. Vegetables, fruits, and risk of colorectal cancer: a case-control study from Uruguay. *Nutrition and Cancer*. 25(3): 297-304.
- Di Pietro, P.F., N.I. Medeiros, F.G. Vieira, M.A. Fausto and A. Bello-Klein. 2007. Breast cancer in southern Brazil: association with past dietary intake. *Nutricion Hospitalaria*. 22(5): 565-572.
- Doll, R. and R. Peto. 1981. The causes of cancer: quantitative estimates of avoidable risks of cancer in the United States today. *Journal of the National Cancer Institute*. 66(6): 1191-1308.
- Duffy, M.J., T.M. Maguire, A. Hill, E. McDermott and N. O'Higgins. 2000. Metalloproteinases: role in breast carcinogenesis, invasion and metastasis. *Breast Cancer Research*. 2: 252-257.



- Eastman, A. and J.R. Rigas. 1999. Modulation of apoptosis signaling pathways and cell cycle regulation. *Seminars in Oncology*. 26(5 Suppl 16): 7-16, discussion 41-42.
- Eberhardt, M.V., C.Y. Lee and R.H. Liu. 2000. Antioxidant activity of fresh apples. *Nature*. 405(6789): 903-904.
- Fairman, M.P. 1990. DNA polymerase delta/PCNA: actions and interactions. *Journal of Cell Science*. 95(Pt 1): 1-4.
- Freedman, L.S., C. Clifford and M. Messina. 1990. Analysis of dietary fat, calories, body weight, and the development of mammary tumors in rats and mice: a review. *Cancer Research*. 50: 5710-5719.
- Gallus, S., R. Talamini, A. Giacosa, M. Montella, V. Ramazzotti, S. Franceschi, E. Negri and C. La Vecchia. 2005. Does an apple a day keep the oncologist away? *Annals of Oncology*. 16(11): 1841-1844.
- Gavrieli, Y., Y. Sherman and S.A. Ben-Sasson. 1992. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *Journal of Cell Biology*. 119(3): 493-501.
- Gosse, F., S. Guyot, S. Roussi, A. Lobstein, B. Fischer, N. Seiler and F. Raul. 2005. Chemopreventive properties of apple procyanidins on human colon cancer-derived metastatic SW620 cells and in a rat model of colon carcinogenesis. *Carcinogenesis*. 26(7): 1291-1295.
- Gosse, F., S. Roussi, S. Guyot, A. Schoenfelder, A. Mann, J.P. Bergerat, N. Seiler and F. Raul. 2006. Potentiation of apple procyanidin-triggered apoptosis by the polyamine oxidase inactivator MDL 72527 in human colon cancer-derived metastatic cells. *International Journal of Oncology*. 29(2): 423-428.
- Gratzner, H.G. 1982. Monoclonal antibody to 5-bromo- and 5-iododeoxyuridine: A new reagent for detection of DNA replication. *Science*. 218(4571): 474-475.
- Griffin, A.C. 1980. Cancer chemoprevention. *Journal of Cancer Research and Clinical Oncology*. 98(1): 1-7.

- Hall, P.A. and D.A. Levinson. 1990: Review: Assessment of cell proliferation in histological material. *Journal of Clinical Pathology*. 43(3): 184-192.
- He, X. and R.H. Liu. 2008. Phytochemicals of apple peels: isolation, structure elucidation, and their antiproliferative and antioxidant activities. *Journal of Agricultural and Food Chemistry*. 56(21): 9905-9910.
- Helin, K. 1998. Regulation of cell proliferation by the E2F transcription factors. *Current Opinion in Genetics and Development*. 8(1): 28-35.
- Hiu, R., G.L. Finney, J.S. Carroll, C.S. Lee, E.A. Musgrove and R.L. Sutherland. 2002. Constitutive overexpression of cyclin D1 but not cyclin E confers acute resistant to antiestrogens in T-47D breast cancer cells. *Cancer Research*. 62(23): 6916-6923.
- Huang, J.W., C.W. Shiau, Y.T. Yang, S.K. Kulp, K.F. Chen, R.W. Brueggemeier, C.L. Shapiro and C.S. Chen. 2005. Peroxisome proliferator-activated receptor gamma-independent ablation of cyclin D1 by thiazolidinediones and their derivatives in breast cancer cells. *Molecular Pharmacology*. 67(4):1342-1348.
- Huerta, S., E.J. Goulet, S. Huerta-Yepez and E.H. Livingston. 2007. Screening and detection of apoptosis. *Journal of Surgical Research*. 139(1): 143-156.
- Hynes, N.E. and H.A. Lane. 2005. ERBB receptors and cancer: the complexity of targeted inhibitors. *Nature Reviews Cancer*. 5(5): 341-354.
- Ip, C., M. Singh, H.J. Thompson and J.A. Scimeca. 1994. Conjugated linoleic acid suppresses mammary carcinogenesis and proliferative activity of the mammary gland in the rat. *Cancer Research*. 54(5): 1212-1215.
- Ishii, Y., A. Pirkmaier, J.V. Alvarez, D.A. Frank, I. Keselman, D. Logothetis, J. Mandeli, M.H. O'Connell, S. Waxman and D. Germain. 2006. Cyclin D1 overexpression and response to bortezomib treatment in a breast cancer model. *Journal of the National Cancer Institute*. 98(17):1238-1247.
- Jemal, A., R. Siegel, E. Ward, Y. Hao, J. Xu, T. Murray, M.J. Thun. 2008. Cancer Statistics, 2008. *CA: A Cancer Journal for Clinicians*. 58(2): 71-96.

- Jeong, J.H., J.Y. An, Y.T. Kwon, L.Y. Li and Y.J. Lee. 2008. Quercetin-induced ubiquitination and down-regulation of Her-2/new. *Journal of Cellular Biochemistry*. 105(2): 585-595.
- Kitagawa, H. and M. Noguchi. 1994. Comparative effects of piroxicam and esculetin on incidence, proliferation, and cell kinetics of mammary carcinomas induced by 7,12-dimethylbenz[a]anthracene in rats on high- and low-fat diets. *Oncology*. 51(5): 401-410.
- Langenfeld, J., H. Kiyokawa, D. Sekula, J. Boyle and E. Dmitrovsky. 1997. Posttranslational regulation of cyclin D1 by retinoic acid: a chemoprevention mechanism. *Proceedings of the National Academy of Sciences USA*. 94(22): 12070-12074.
- Lin, C.W., W.C. Hou, S.C. Shen, S.H. Juan, C.H. Ko, L.M. Wang and Y.C. Chen. 2008. Quercetin inhibition of tumor invasion via suppressing PKC delta/ERK/AP-1-dependent matrix metalloproteinase-9 activation in breast carcinoma cells. *Carcinogenesis*. 29(9): 1807-1815.
- Liu, J.R., H. W. Dong, B.Q. Chen, P. Zhao and R.H. Liu. 2009. Fresh apples suppress mammary carcinogenesis and proliferative activity and induce apoptosis in mammary tumors of the Sprague-Dawley rat. *Journal of Agricultural and Food Chemistry*. 57(1): 297-304.
- Liu, M., X.Q. Li, C. Weber, C.Y. Lee, J. Brown and R.H. Liu. 2002. Antioxidant and antiproliferative activities of raspberries. *Journal of Agricultural and Food Chemistry*. 50(10): 2926-2930.
- Liu, R.H. 2003. Health benefits of fruit and vegetables are from additive and synergistic combinations of phytochemicals. *American Journal of Clinical Nutrition*. 78(3 Suppl): 517S-520S.
- Liu, R.H. 2004. Potential synergy of phytochemicals in cancer prevention: mechanism of action. *Journal of Nutrition*. 134(12 Suppl): 3479S-3485S.
- Liu, R.H. and J. Sun. 2003. Antiproliferative activity of apples is not due to phenolic-induced hydrogen peroxide formation. *Journal of Agricultural and Food Chemistry*. 51(6): 1718-1723.

- Liu, R.H., J. Liu and B. Chen. 2005. Apples prevent mammary tumors in rats. *Journal of Agricultural and Food Chemistry*. 53(6): 2341-2343.
- Maldonado-Celis, M.E., S. Roussi, C. Foltzer-Jourdainne, F. Gosse, A. Lobstein, C. Habold, A. Roessner, R. Schneider-Stock, and F. Raul. 2008. Modulation of polyamines of apoptotic pathways triggered by procyanidins in human metastatic SW620 cells. *Cellular and Molecular Life Sciences*. 65(9): 1425-1434.
- McCormick, D. and P.A. Hall. 1992. The complexities of proliferation cell nuclear antigen. *Histopathology*. 21(6): 591-594.
- Michels, K.B., E. Giovannucci, A.T. Chan, R. Singhanian, C.S. Fuchs and W.C. Willett. 2006. Fruit and vegetable consumption and colorectal adenomas in the Nurses' Health Study. *Cancer Research*. 66(7): 3942-3953.
- Moolgavkar, S.H. 1978. The multistage theory of carcinogenesis and the age distribution of cancer in man. *Journal of the National Cancer Institute*. 61(1): 49-52.
- Olayioye, M.A., R.M. Never, H.A. Lane and N.E. Hynes. 2000. The ErbB signaling network: receptor heterodimerization in development and cancer. *EMBO Journal*. 19(13): 3159-3167.
- Oliveria, S.A., P.J. Christos and M. Berwick. 1997. The role of epidemiology in cancer prevention. *Proceedings of the Society for Experimental Biology and Medicine*. 216(2): 142-150.
- Roussi, S., F. Gosse, D. Aoude-Werner, X. Zhang, E. Marchioni, P. Geoffroy, M. Miesch and F. Raul. 2007. Mitochondrial perturbation, oxidative stress and lysosomal destabilization are involved in 7 $\beta$ -hydroxysteroid and 7 $\beta$ -hydroxycholesterol triggered apoptosis in human colon cancer cells. *Apoptosis*. 12(1): 87-96.
- Sanchez, I. and B.D. Dynlacht. 1996. Transcriptional control of the cell cycle. *Current Opinion in Cell Biology*. 8(3): 318-324.
- Sherr, C.J. 1995. D-type cyclins. *Trends in Biochemical Sciences*. 20(5): 187-190.
- Slattery, M.L. 2001. Does an apple a day keep breast cancer away? *Journal of the American Medical Association*. 285(6): 799-801.

- Sun, J. and R.H. Liu. 2008. Apple phytochemical extracts inhibit proliferation of estrogen-dependent and estrogen-independent human breast cancer cells through cell cycle modulation. *Journal of Agricultural and Food Chemistry*. 56(24): 11661-11667.
- Sun, J., Y.F. Chu, X. Wu and R.H. Liu. 2002. Antioxidant and antiproliferative activities of common fruits. *Journal of Agricultural and Food Chemistry*. 50(25): 7449-7454.
- Tsao, R., R. Yang, J.C. Young and H. Zhu. 2003. Polyphenolic profiles in eight apple cultivars using high-performance liquid chromatography (HPLC). *Journal of Agricultural and Food Chemistry*. 51(21): 6347-6353.
- Veeriah, S., C. Miene, N. Habermann, T. Hofmann, S. Klenow, J. Sauer, F. Bohmer, S. Wolfl and B.L. Pool-Zobel. 2008. Apple polyphenols modulate expression of selected genes related to toxicological defense and stress response in human colon adenoma cells. *International Journal of Cancer*. 122(12): 2647-2655.
- Whitsett, T., M. Carpenter and C.A. Lamariniere. 2006. Resveratrol, but not EGCG, in the diet suppresses DMBA-induced mammary cancer in rats. *Journal of Carcinogenesis*. 5:15.
- Willett, W.C.. 2002. Balancing life-style and genomics research for disease prevention. *Science*. 296: 695-698.
- Wolfe, K., X. Wu and R.H. Liu. 2003. Antioxidant activity of apple peels. *Journal of Agricultural and Food Chemistry*. 51(3): 609-614.
- Yoon, H. and R.H. Liu. 2007. Effect of selected phytochemicals and apple extracts on NF- $\kappa$ B activation in human breast cancer MCF-7 cells. *Journal of Agricultural and Food Chemistry*. 55(8): 3167-3173.
- Yu, Q., Y. Geng and P. Sicinski. 2001. Specific protection against breast cancers by cyclin D1 ablation. *Nature*. 411(6841): 1017-1021.